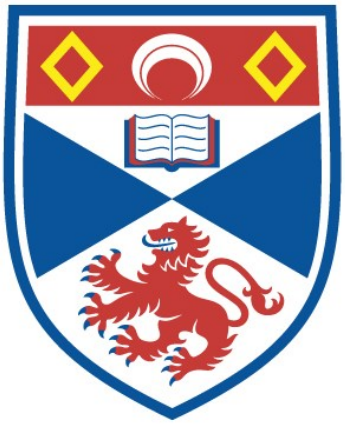
RNA SYNTHESIS, MOVEMENT AND CYTOPLASMIC MICROTUBULES IN THE TELETROPHIC OVARY OF NOTONECTA GLAUCA GLAUCA

Howard Stebbings

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1972

RNA SYNTHESIS, MOVEMENT, AND CYTOPLASMIC MICROTUBULES, IN THE TELOTROPHIC OVARY OF NOTONECTA GLAUCA GLAUCA (Linn).

A Thesis presented for the degree of Doctor of Philosophy

to the University of St. Andrews

by Howard Stebbings, B.Sc.

Department of Zoology, University of St. Andrews,

St. Andrews, Fife, Scotland

and

Department of Zoology, University of Leicester,

Leicester, England.

DECLARATION.

I hereby declare that this thesis is based upon the results of my own work. Professor H.C. Macgregor and I are jointly responsible for Appendix I. Appendix III is the account of a study by myself and Dr. N.A. Ratcliffe; and Appendix IV a study by myself and Mr. J.H.M. Willison.

My thesis has not been submitted for any other degree.

HOWARD STEBBINGS

CERTIFICATE.

I certify that Howard Stebbings has spent nine terms investigating RNA synthesis, movement, and cytoplasmic microtubules in the telotrophic ovary of *Notonecta glauca glauca* Linn., that he has fulfilled the conditions of the Ordinance and regulations and that he is qualified to submit this thesis for the degree of Doctor of Philosophy.

H.G. CALLAN

UNIVERSITY CAREER AND RESEARCH EXPERIENCE.

I entered the University of St. Andrews in October, 1965, and was awarded B.Sc. Hons. II_1 in Zoology in June, 1969. During my final year as an undergraduate student, I commenced my investigation into the synthesis and transport of RNA in telotrophic ovarioles.

In September, 1969, I was awarded an S.R.C. Research Studentship, and was admitted as a research student in the University of St. Andrews, under Ordinance General No. 12, and as a candidate for the degree of Ph.D. under Ordinance No. 88.

Since September, 1969, I have continued with the examination of telotrophic ovaries. I have investigated the structure of the telotrophic ovary of Notonecta, an aquatic hemipteran, and looked at the pattern of synthesis of RNA within the ovary. I have shown that there is a system of microtubules associated with the movement of ribosomes within the ovary.

In the late summer of 1970, I transferred to the Department of Zoology, University of Leicester, along with Professor H.C. Macgregor, my supervisor, and have since concentrated on examining the substructure of microtubules and the action of antimitotic agents on these organelles.

I was awarded a Short Term Fellowship by the European Molecular Biology Organisation, in September, 1970, to visit the laboratory of Dr. Joel Rosenbaum for one term, where I learnt techniques for the isolation and characterisation of microtubule proteins, and also collaborated in examining the effects of an antimitotic drug on the structure of microtubules.

The up-to-date results of my research are presented here as a thesis for the degree of Doctor of Philosophy.

ACKNOWLEDGEMENTS.

I am grateful to the numerous people who have helped and advised me during my research training.

I thank Professor H.G. Callan and Dr. J.B. Tucker (Department of Zoology, St. Andrews), and Dr. N.A. Ratcliffe (Department of Zoology, Leicester) for their helpful advice and discussion. I thank the technical staff of the Zoology Departments of both St. Andrews and Leicester Universities; especially Mr. J.B. Mackie for his initial instruction in electron microscopy. I am also grateful to Mr. Mackie and Mr. D. Cunningham for helping me to obtain specimens for my research.

I greatly value the time spent with Dr. Joel Rosenbaum (Biology Department, Yale University, U.S.A.), and the members of his laboratory, and I thank them for the instruction which they gave me.

Mr. J.H.M. Willison (Department of Botany, University of Nottingham) has taught me the technique of freeze-etching, and we are at present collaborating in a number of projects.

I thank especially Professor H.C. Macgregor for his instruction, advice, interest and general guidance during my training as a research student. I shall always consider myself extremely fortunate to have been trained by a scientist with such a high standard of research, and above all by someone who has unending patience with, and total commitment to his students.

Finally, I thank the Science Research Council for the Research Studentship, which I have held for the past three years.

CONTENTS

INTRODUCTION

In this study, I set out to examine a number of features of the ovary of the aquatic insect, Notonecta glauca glauca Linn. In addition to investigating the structure of the ovary, and the pattern of synthesis of RNA within the ovary during oogenesis, I wished to evaluate the contribution which the different types of cells within the ovary make to the growing oocytes.

Female insects have two ovaries, each composed of egg tubes or ovarioles. The number of ovarioles in each ovary is characteristic of a species and varies from few to many (Wigglesworth, 1953). In some Diptera, for example, there may be only one ovariole per ovary, while in some Isopterans there are over a thousand.

Insect ovaries have been classified into two types (Fig. 1), depending on the presence or absence of nutritive cells within the ovary. Nutritive cells, which are sometimes referred to as trophic or nurse cells, are supplementary cells which contribute to the oocytes during oogenesis. Panoistic ovaries have no nutritive cells and are found in the older orders of insects - the Thysanura, Orthoptera, Isoptera, Odonata and Plecoptera (see review Bonhag, 1958). Meroistic ovaries, on the other hand, do have nutritive cells, and are further sub-divided into polytrophic types, in which a nutritive cell or a group of nutritive cells alternates with single oocytes down the ovariole, and telotrophic types, in which the nutritive cells are concentrated in a distinct trophic region at the anterior of the ovariole, and are usually joined to the oocytes by cytoplasmic cords.

Several workers have investigated the origins of the different types of cells found in insect ovaries, and concluded that the oocytes and the nutritive cells are products of the germ line, while all other ovarian

tissue is of mesodermal origin (Wieman, 1910; Nelson, 1934; Wick & Bonhag, 1955).

In insects with panoistic ovaries, all the oogonia develop into oocytes. In the meroistic ovary of Drosophila melanogaster, Koch, Smith & King (1967) have studied the morphogenesis of the egg chamber and demonstrated how the products of a single oogonial cell develop into a branching chain of 16 cells which are interconnected. They also suggest the mechanism by which a) cell division is inhibited once 16 cells are formed, b) the future cleavage planes of the cell are programmed, and c) the way in which the oocyte is differentiated from the nutritive cells.

In polytrophic ovaries, there is, with the exception of one family within the Diptera (Matuszewski, 1968), a distinct ratio of nutritive cells to oocytes in each follicle. For example, in the seaside earwig Anisolabis maritima, there is one nutritive cell to each oocyte (Kornhauser, 1930), while in the great diving beetle, Dytiscus marginalis, there are fifteen nutritive cells to every oocyte (Giardina, 1901). The morphology of the telotrophic ovary makes it more difficult to determine its ratio of nutritive cells to oocytes.

The contribution of the nutritive cells to the oocytes has been a topic of much speculation and research, and most of the experimental work has been with polytrophic ovaries. Peacock & Gresson (1928), working with sawflies, described how the cell boundaries of the nutritive cells become indistinct in the older follicles, and how some of the cytoplasm, together with contained accessory nuclei, pass through a narrow channel into the oocyte, where the nuclei become engulfed and absorbed in the ooplasm.

The possibility that nucleic acids are passed from nutritive cells to the oocytes of insects was postulated before reliable histochemical

techniques were in use (Wieman, 1910; Bhandari & Nath, 1930). According to Wieman (1910) in the telotrophic ovary of the beetle, Leptinotarsa signaticollis, a steady stream of nutritive material, in the form of basic staining granules, passes into the egg by way of the "egg-string".

Kaufmann, McDonald, Bernstein, von Borstel & Das (1953) have investigated the wasp, Habrobracon, and reported that DNA passes from the nutritive cells to the oocytes, where it is broken down and dispersed.

Two authors have presented circumstantial evidence which suggests that in the honey bee, RNA passes from nutritive cells to oocytes (Morgenthaler, 1952; Bier, 1954). Sirlin & Jacob (1960) fed female Drosophila melanogaster for 3h with food containing ^{14}C-orotic acid, which is a precursor of RNA. They then located the tracer by autoradiography. From their autoradiographs, they discovered that the tracer moves, with time, along streams of ooplasm which are continuous with the cytoplasm of nutritive cells, and they interpreted this as indicating a flow towards the oocytes.

In a similar experiment, Bier (1963) demonstrated the passage of labelled RNA from the nutritive cells to the oocytes in the polytrophic ovary of Musca domesticus. He did not, however, find an accumulation of tracer in the oocytes, and concluded that the RNA had a high turnover rate and that this was probably messenger RNA.

Pollack & Telfer (1969), who worked with Cecropia moths, studied autoradiographs of follicles labelled with ^{3}H-uridine. As in other polytrophic ovaries, they found that the nutritive cells were the most conspicuous source of oocyte RNA. They did observe, however, that in early previtellogenic follicles, the germinal vesicle incorporated ^{3}H-uridine, while in vitellogenic and later stages they could detect no germinal vesicle or follicle cell contribution to oocyte RNA.

In the polytrophic ovaries which have been studied there is a synthesis of RNA within the nutritive cells and then a passage of this RNA to the oocytes. What type of RNA is this? Electron micrographs of the nutritive cells of a number of insects has shown that their cytoplasm is rich in ribosomes (King, 1960; Roth & Porter, 1964; King & Aggarwal, 1965), and Roth, Freilinger & Deborde (1968) have analysed the RNA produced in the intact ovaries of mosquitoes and found that ribosomal and transfer RNAs are the predominant species. More conclusively, Hughes & Berry (1970), investigating the giant silk moth, Antheraea polyphemus, have developed a method for separating and isolating the three types of cells which make up the ovarian follicle. Using this technique, they were able to analyse the RNA produced by the nutritive cells, and have shown that the majority of this was ribosomal.

The degree of involvement of the germinal vesicle in RNA synthesis varies widely in different insects. In orders with panoistic ovaries, such as the Orthoptera, multiple nucleoli have been seen in the germinal vesicle (Bier, Kunz & Ribbert, 1967; Lima-de-Faria, Nilsson, Cave, Puga & Jaworska, 1968; Kunz, 1969), and these, together with conspicuous lampbrush chromosomes have been found to incorporate ^{3}H-uridine into RNA (Bier, Kunz & Ribbert, 1967). Indeed, in panoistic ovaries, the germinal vesicle is the only documented source of cytoplasmic RNA.

In the meroistic ovaries of several Dipterans and adephagous Coleopterans, Bier, Kunz & Ribbert (1967) detected incorporation of ^{3}H-uridine into chromosomal RNA of the germinal vesicle. In these cases, the development of lampbrush chromosomes was reduced, and the intensity of labelling was correspondingly low, in comparison with the nutritive cells.

Although in the majority of polytrophic ovaries studied, the nutritive cells are the major source of RNA for the oocytes, some exceptions have been found. The germinal vesicles of dytiscid beetles have a body of extrachromosomal DNA, first demonstrated by Giardina (1901). Urbani & Russo-Caia (1964) have studied the synthesis of nucleic acids in the ovaries of several beetles and have shown that oocytes and nutritive cells both incorporate ^{3}H-uridine into RNA, and Bier, Kunz & Ribbert (1967), working with Dytiscus, have demonstrated that the extra DNA in the oocytes is involved in the production of nucleolar RNA. Their autoradiographs indicate that RNA from the nutritive cells and from the germinal vesicle enters the ooplasm. Hybridization experiments (Gall, Macgregor & Kidston, 1969) have shown that there is an amplification of ribosomal DNA in the ovary, although this rDNA does not account for all the extra DNA in the ovary. A similar situation has been found in Tipula (Lima-de-Faria & Moses, 1966).

A number of workers have investigated telotrophic ovaries. Bhandari & Nath (1930) looked at the Red Cotton Bug, Dysdercus cingulatus, and found that the oocytes were connected to the nutritive cells by bundles of fine "roots", so called because of their striated appearance. In speculating as to the function of the connections, they suggested that the nutritive cells disintegrate and their contents pass along the roots as a liquid. They single out the Golgi elements of the nutritive cells in particular, and propose that these flow down the roots and enter the eggs, reappearing in the oocytes in the form of thick hyaline gelatinous fibres, which spread out through the cytoplasm.

Schrader & Leuchtenberger (1952) have looked at Acanthocephala bicoloripes, and Bonhag (1955) at Onchopeltus fasciatus, another Hemipteran,

and both described a situation in which DNA is extruded in globular form from the disintegrating nuclei of the nutritive cells. This DNA, they propose, loses its stainability with Feulgen reagent and is passed into the trophic core, and thence down the nutritive tubes to the oocytes. In his report, Bonhag (1955) also described flow-like patterns of RNA extending from the cytoplasm around the nutritive cell nuclei, and into the trophic core.

There is evidence therefore that nutritive cells contribute to the oocytes in both polytrophic and telotrophic ovaries, and reports that the nutritive cells in different species become polyploid (Painter & Reindorp, 1939; Bier, 1957). According to Schultz (1956), in Drosophila, the nutritive cells contain DNA ranging from the 16C - 1024C amounts. Hsu & Hansen (1953) have shown that nutritive cell chromosomes increase in breadth and length, while King, Rubinson & Smith (1956) also working with Drosophila, have reported that the chromosomes of the nutritive cells elongate and spread out in the nuclei, until, in the largest cells, lampbrush loops appear to originate from the chromosomal axes. They suggest that as the chromosomes elongate, they become polytene, and banded polytene chromosomes have been seen in the nutritive cells of adults of 16 species of Diptera (Stalker, 1954). Basile (1969) has also described this phenomenon in the nutritive cells of Rhynchosciara angelae and suggested that the change in the morphology of the chromosomes from active cryptopolytene to a partially condensed state (typically polytene) could indicate a difference in the kind and quantity of RNA produced at these two stages.

I decided to investigate the telotrophic ovary, paying special attention to the nature and function of the nutritive cells and the connections between the nutritive cells and the oocytes. I wished especially

to evaluate the contribution of the different types of cells within the ovary, to the growing oocytes.

During the course of my experiments, I discovered that the nutritive tubes were packed with cytoplasmic microtubules. Microtubules have been found in a wide variety of animal and plant cells, since the introduction in the early 1960s of improved fixatives for electron microscopy. The mass of microtubules within limited tracts in the telotrophic ovary have made it ideal for a study of the biology of these organelles.

I have examined the structure of microtubules and the spatial relationship of microtubules to other cytoplasmic inclusions. I have looked at thin sectioned and negatively stained preparations of ovarian tissue and compared the observations from these techniques, with information gained from freeze-etched replicas of ovaries, where the tissue had not been subjected to chemical fixation. A further large section of this work concerns a study of the effects of various anti-mitotic treatments on the microtubules. Such information increases the value of antimitotic treatments as tools for answering questions about the stability, function, composition, assembly and breakdown of microtubules.

I have employed a wide variety of techniques and approaches during my investigations of the telotrophic ovary. Accordingly, I decided to present the materials, methods and observations, relating to each technique, in the approximate chronological order in which they were performed (Sections I, II & III). This is followed by a co-ordinated discussion of all my observations.

MATERIALS, METHODS & OBSERVATIONS

Notonecta glauca glauca Linn. were caught in a fire pond in Tentsmuir Forest (O.S. map ref. No. 473212) and in the Curling Pond at Boarhills (No. 548134), both in Fife, Scotland. Large numbers of adult Notonecta could be caught in both ponds in the Autumn, but the numbers decreased throughout the Winter, and adults were rarely found after June. Both the Tentsmuir and Boarhills ponds contained a considerably larger population of aquatic Hemipterans and Coleopterans than either the canal or the many ponds in Leicestershire, which I have fished. The eggs of Notonecta are laid in early spring. The first naiads emerge during May and June in Scotland, and develop into adult forms by early Autumn.

To ensure a steady supply, I kept Notonecta adults in the laboratory in freshwater tanks for up to three weeks. By keeping pond weed in the tanks, and maintaining a food supply of Tubifex, I was able to prevent them preying on each other.

To remove the ovaries, decapitated animals were pinned, dorsal side up, in Locke's insect Ringer* and the tergites removed with watchmaker's forceps, using a Carl Zeiss Stereomicroscope. Each ovary stretched the length of the abdomen, and consisted of a terminal filament, a germarium, a vitellarium, and at the posterior, the seven ovarioles fused into a lateral oviduct.

*Locke's insect Ringer was used throughout this study, and consisted of:

9.00 g	NaCl	per litre of distilled water
0.42 g	KCl	
0.25 g	$CaCl_2$	
0.20 g	$NaHCO_3$	
2.50 g	$C_6H_{12}O_6$ (glucose)	

STRUCTURE AND FUNCTION OF TELOTROPHIC OVARIES

(a) CYTOCHEMISTRY OF OVARIOLES AND NATURE OF NUTRITIVE CELLS.

I commenced this study by an examination of the cytochemistry of the ovaries using gallocyanine-chrome alum and Feulgen stain.

Gallocyanine-chrome alum sections:

This stain was used, as it is simple, reliable and specific under certain conditions for nucleic acids. I distinguished the RNA from DNA by comparing preparations which had been preincubated in ribonuclease (RNase), with untreated preparations. Freshly removed ovaries were fixed in 3 : 1 alcohol : acetic acid (Clarke, 1851) for three hours and embedded in wax (M.Pt.55°C). Sections 7µm thick were cut using an M.S.E. rotary microtome and mounted on clean slides. The wax was removed in xylene (1h with continuous stirring) and the sections hydrated through an alcohol series to water. Sections were stained in gallocyanine-chrome alum (G.T. Gurr, London) (per Swift, 1955) for 48h. They were passed quickly through an alcohol series, dehydrated in 100% alcohol (2 changes) and mounted in Canada balsam. Other sections were treated with a solution of RNase (0.2mg/ml RNase 1 Sigma in 0.01M phosphate buffer pH 6.9) for 2h at 37°C. Control preparations were incubated in phosphate buffer only.

Feulgen stained sections:

I used Feulgen stain to determine the precise distribution of DNA in the ovaries of Notonecta, while bearing in mind that both Schrader and Leuchtenberger (1952) and Bonhag (1955) had suggested that the nutritive cells exuded DNA droplets which became Feulgen-negative, before being passed down the nutritive tubes. Ovaries were fixed, embedded and sectioned as for gallocyanine-chrome alum preparations. The wax was removed in xylene and sections hydrated by passing them through an alcohol series to water. The sections were hydrolized in 1N HCl at 60°C for 10m, and then stained in Feulgen reagent (per Swift, 1955) for 2 h. They were then washed in "SO_2 water" per Swift 1955 for 30m (2 changes), dehydrated in an alcohol series, cleared in xylene and mounted in Canada balsam.

Feulgen stained squash preparations:

Ovaries were transferred to 45% acetic acid after 1h fixation in 3 : 1 alcohol : acetic acid. The trophic regions from single ovarioles were removed and placed in a drop of 45% acetic acid on a slide and squashed with varying degrees of pressure between folded filter paper. Cover glasses were removed by the "dry ice" method. The slides were placed in 95% alcohol and hydrated through an alcohol series. They were then hydrolized in 1N HCl at 60°C for 10m, rinsed in water and stained in Feulgen reagent for 2h. The preparations were rinsed in SO_2 water for 45m, dehydrated in alcohol, cleared in xylene and mounted in Zeiss immersion oil.

At the anterior of each ovariole is a trophic region approximately 0.7mm long and 0.3mm wide (Fig. 2). It consists of nutritive cells arranged around a central trophic core (Fig. 3). The nuclei of these cells have large nucleoli. About twenty nutritive tubes pass back from the trophic core, around tightly packed prefollicular tissue and developing oocytes, and each supplies one oocyte in the ovariole. The anterior oocytes are spherical, but the posterior oocytes are larger and elongated, and are no longer supplied by a tube. The cytoplasm of the trophic core and the nutritive tubes show 'hair-like' wavy striations along their length (Fig. 4).

Transverse sections through ovarioles show that the tubes are round or oval in cross section and vary from 5 - 20μm in width. The oocytes and nutritive tubes are surrounded by follicle tissue. Several layers of follicle cells surround the younger oocytes, while older oocytes are enclosed by a single layer of cells. These follicle cells are binucleate, and each nucleus has a large nucleolus. The ramifications around the nutritive cell nuclei, the trophic core, the cytoplasm of the nutritive tubes and the oocytes all stained strongly with gallocyanine (Fig. 2).

In sections which had been treated with RNase before staining, nothing stained other than nuclear chromatin.

The Feulgen preparations demonstrated the great difference in size and structure of the trophic nuclei. At the anterior tip of the trophic region, the nuclei were approximately 5μm in diameter, with Feulgen-positive granules around their periphery. Further back, the nuclei measured about 20μm in diameter (Fig. 6). These largest trophic nuclei have a large Feulgen-negative area, the nucleolus, bordered by two large masses of Feulgen-positive material. In squash preparations which have been pressed firmly, the nucleus occasionally bursts open and the large heterochromatic

masses are themselves compressed. In many preparations treated in this way, distinct Feulgen-positive loops of varying sizes could be seen emerging from the heterochromatin (Figs. 8,9, 10 & 11).

The trophic core and the nutritive tubes are Feulgen-negative, (Fig.5). No part of the oocyte nucleus was distinctly Feulgen-positive, (Fig.7).

It was clear from the Feulgen squashes, that the nutritive cells contained different amounts of DNA, raising the question as to how the extra DNA in the larger cells was produced. Was the extra DNA the result of selective amplification of a particular portion of the genome, or was it merely a consequence of polyploidy?

A high density indicates a high guanine-cytosine (G + C) content in a DNA sample. rDNA is G + C rich, has a high buoyant density, and can therefore be separated from the remainder of the chromosomal DNA, which has a relatively low G + C content, by density gradient centrifugation in CsCl. By exploiting this situation and by employing techniques of nucleic acid hybridisation, Gall (1968), Brown and Dawid (1968), and Evans and Birnstiel (1968) were able to show enrichment of rDNA in the germinal vesicles of amphibian oocytes, brought about by a process of ribosomal gene amplification (see review, Macgregor, 1972).

To investigate the nature of the extra DNA within the large nutritive cells of Notonecta, I decided to extract the DNA from nutritive cells, and to centrifuge this to equilibrium on CsCl gradients, to observe its density profile.

Fractionation of the DNA of nutritive cells on CsCl gradients:

Ovaries were removed from 40 animals and their trophic regions cut off and stored in 70% ethanol. The trophic tissue was gently ground in an homogeniser in 70% ethanol and centrifuged

for 20m at 5,000 r.p.m. in a Sorvall H B-4 rotor. The tissue was resuspended in 2.0 ml of standard saline citrate (SSC) (0.15M NaCl + 0.015M sodium citrate, pH 7.0, Marmur, 1961). To this trophic tissue extract was added 50µl pronase solution (1mg/ml pronase) and 0.2ml of 20% sodium lauryl sulphate (SLS). The mixture was incubated overnight at 37°C. An equal volume of phenol, saturated with 0.1 x SSC, was added to the sample to precipitate the protein and the mixture was shaken for 10m and allowed to stand for an hour. The mixture was centrifuged at 10,000 r.p.m. for 20m in a Sorvall H B-4 rotor. The aqueous layer, containing the DNA was pipetted off. DNA was precipitated by adding $2\frac{1}{2}$ volumes of absolute ethanol. The fibrous precipitate was collected by centrifugation at 10,000 r.p.m. for 20m, washed repeatedly with 70% ethanol and redissolved in 2ml, 0.1 SSC. RNase "A" (100µg/ml in SSC) and 10µg RNase "T_1", were added and the mixture was incubated for 1h at 37°C. The DNA was reprecipitated by adding 1/10 volume 1M NaCl and $2\frac{1}{2}$ volumes of ethanol. The precipitate was collected by centrifuging at 10,000 r.p.m. for 15m and redissolved in 1ml 0.1 x SSC.

The optical density at 260mµ wavelength ($O.D._{260}$) of the sample was read. A solution of 50µg/ml DNA has an $O.D._{260}$ of 1.0. This sample had an $O.D._{260}$ of 0.72 and therefore contained 50 x 0.72 = 36µg/ml of DNA.

To check the purity of the DNA sample, the optical densities at 220, 232 and 280mµ were also read. For a pure DNA sample, the $O.D._{280}$ should approximate to the $O.D._{232}$ and their value should

be about half of that at O.D.$_{260}$. The O.D.$_{220}$ should not be greater than the O.D.$_{260}$. These tests showed that the DNA sample was satisfactory.

The DNA solution was mixed with saturated (25°C) optical grade CsCl (Harshaw Chemical Inc.) in the proportions 6.420g CsCl + 1.000g of DNA + 0.165g of 0.1 x SSC. These proportions were selected to give a mean density of 1.678 cm^{-3}. The mixture was placed in a 10ml propylene tube and centrifuged at 41,500 r.p.m. at 15°C for 22h in an M.S.E. 10 x 10ml angle rotor.

The sample was removed from the centrifuge, the bottom of the tube pierced and '12 drop' fractions collected. 200µl 0.1 x SSC were added to each and the O.D.$_{260}$ of each fraction was measured.

As can be seen from Fig.12 the DNA from the trophic nuclei formed a single band when centrifuged to equilibrium in CsCl. There was no evidence of a separate band of DNA of high density, as found when the ribosomal DNA was selectively amplified in amphibian oocytes. It seemed most likely therefore, that the variation in the amounts of DNA in the nuclei of the nutritive cells was a consequence of polyploidy.

Microspectrophotometry of Feulgen stained squash preparations of nutritive cells.

The degree of ploidy was estimated by measuring the Feulgen dye content of nuclei in squash preparations. This was done using a Vickers M85 Integrating Microdensitometer. This instrument scans an area, the shape and size of which can be easily adjusted. The wavelength chosen was near to the maximum absorbance of Feulgen

stain, and masks of the appropriate sizes were manoeuvred around selected nuclei. The Feulgen dye content of all sizes of nuclei was measured and compared with the measurements of the diploid nuclei of ovariole sheath cells. A numerical value for each nucleus was obtained.

In addition to measuring the degrees of ploidy of the nutritive cells, I decided to measure the Feulgen dye content of the largest trophic nuclei and that of the two distinct Feulgen-positive masses within these nuclei. In this way, it was possible to estimate what proportion of the DNA of these cells was present as heterochromatin. I thought that this information might help in understanding the nature and function of this heterochromatin DNA

The Feulgen dye content of the different sizes of nuclei measured are shown in Table 1. Measurements were made on single nuclei within the trophic region, until 8 nuclei from each class had been measured. Calculation of the mean Feulgen dye content of 8 nuclei from each class showed that this value approximately doubled from one class to the next, and that the largest nuclei contained approximately 140 times as much DNA as the smallest cells - which I have judged to be diploid.

The proportion of DNA present in the two heterochromatic masses within a particular nutritive cell was estimated by measuring the Feulgen dye content of the two masses, summing them, and then expressing this as a percentage of the total Feulgen dye content of that cell. As can be seen by Table 2, the heterochromatic masses represented approximately 8.0% of the total DNA. These estimations were only carried out on the largest nuclei, because of the limitations of the technique, and although the

proportion of heterochromatin to total DNA appears to be typical for cells at this particular stage of ploidy, from examination of the Feulgen stained squash preparations, I consider it likely that this proportion varies from stage to stage. Indeed, heterochromatic masses are not discernable in the smallest nuclei.

Table 1

Table showing the Feulgen dye content of ovariole sheath cells, and 7 classes of trophic nuclei. Eight nuclei from each class were measured and the mean value of these measurements is given. (The figures are arbitrary units).

	Ovariole sheath cells	Classes of trophic nuclei						
		1	2	3	4	5	6	7
1	0.47	0.86	1.28	2.60	6.26	11.96	26.41	58.82
2	0.44	0.84	1.25	2.62	6.41	14.78	27.24	62.70
3	0.34	0.65	1.26	2.17	5.85	12.68	24.90	56.31
4	0.41	0.81	1.47	2.44	6.17	15.39	23.50	57.70
5	0.39	0.69	1.14	2.09	5.90	12.61	22.86	58.88
6	0.46	0.82	1.24	3.07	6.34	11.15	25.22	55.04
7	0.40	0.99	1.47	2.05	5.70	12.72	24.04	61.24
8	0.39	0.84	1.50	2.34	6.10	14.02	26.82	60.90
Mean	0.41	0.81	1.33	2.42	6.09	13.16	25.12	58.95
Standard deviation	±0.004	±0.11	±0.13	±0.34	±0.25	±1.44	±1.60	±2.59

Table 2

Table of Feulgen dye content of trophic nuclei and the heterochromatic blobs within these nuclei. The percentage of heterochromatin to total DNA has been estimated from these results. (The figures are arbitrary units).

Feulgen dye content of nucleus A	Feulgen dye content of two heterochromatic masses B	C	Percentage of Feulgen dye in heterochromatin $\frac{B + C}{A} \times 100$
12.11	0.39	0.60	8.2%
15.11	0.65	0.61	9.6
15.88	0.45	0.69	7.2
13.26	0.52	0.59	8.4
14.02	0.48	0.54	9.1

b) SYNTHESIS AND MOVEMENT OF RNA WITHIN OVARIOLES AND LOCATION OF THE RIBOSOMAL GENES IN THE NUTRITIVE CELLS.

As cited in my introduction, a number of authors have speculated on, and in some cases actually demonstrated, the passage of nucleic acids from the nutritive cells to the oocytes. My Feulgen preparations do not show any detectable DNA in the nutritive tubes of Notonecta, and I think it unlikely that DNA passes from the nutritive cells to the oocytes in a Feulgen-negative form, as has been suggested (Schrader & Leuchtenberger, 1952). There are reports, both on the basis of staining with azure B (King, 1964) and autoradiography (Sirlin & Jacob, 1960; Bier, 1963; Pollack & Telfer, 1969) that RNA passes from the nutritive cells to the oocytes - at least in polytrophic ovaries. There is also evidence from electron microscopy (King, 1960; Roth & Porter, 1964; King & Aggarwal, 1965) and RNA analysis (Roth, Freilinger & Debords, 1968) that the majority of the RNA is ribosomal. It is certainly true that oocytes in general store ribosomes during oogenesis (Brown, 1964).

What is the situation in the telotrophic ovary of Notonecta? My experiments have shown that the nutritive cells become polyploid, as do those of many other insects (Painter & Reindorp, 1939; Schultz, 1956; Bier, 1957) and they have a large nucleolus bounded by two distinct heterochromatic blobs. In addition, the area around the nutritive cells, the trophic core, the nutritive tubes, and the cytoplasm of the oocytes were all strongly basophilic.

For these reasons I supposed that the nutritive cells in the telotrophic ovary synthesise RNA, probably ribosomal, which then passes to the oocytes, To investigate this hypothesis I decided to:

a) examine by autoradiography the synthesis and movement of RNA within the ovary.

b) determine the position of the ribosomal genes within the nutritive cells, by in situ cytological hybridisation, to see whether there is anything peculiar about the arrangement of rDNA in these nuclei.

c) look for structures involved with movement of RNA in ovarioles, by means of electron microscopy.

First I examined the incorporation of ^{3}H-uridine into the nutritive cells, oocytes and follicle cells. I also looked for movement of RNA within the ovariole, because of my supposition that RNA passes from the trophic region to the oocytes down the nutritive tubes. To record the movement of labelled RNA it was necessary to look at ovarioles which had been exposed to ^{3}H-uridine for different lengths of time.

Autoradiography after incorporation of ^{3}H-uridine:

Female Notonecta were injected through the arthrodial membrane between the two posterior sternites with 5µl (5µCi) of uridine labelled with tritium (Uridine-T(G) specific activity 1.2 Ci/mM) obtained from the Radiochemical Centre, Amersham. The ovaries were removed at intervals of 2h, 8h, 12h, 24h and 3w after injection and fixed for 24h in Sanfelice's fixative (Darlington & La Cour, 1942). They were then washed in running tap water for 24h and dehydrated through an alcohol series. The ovaries were transferred to a mixture of equal parts absolute alcohol and methacrylate for 2h. They were then put into filtered, degassed, methacrylate mixture for 2h and methacrylate plus catalyst for 18h. The ovaries were surface embedded using a technique first described by Gall (1956). Single ovaries were placed on slides and gelatin capsules, 2/3 full of syrupy pre-polymerised methacrylate, were inverted over each. The slides were then placed in an oven at 70°C for 4h, whereupon the oven was switched off to allow the methacrylate to cool slowly.

The blocks were snapped off the slides, trimmed and 1µm longitudinal and transverse sections were cut with glass knives on a Cambridge Ultramicrotome Mk II and a Porter Blum microtome, and mounted on slides.

The methacrylate was removed from the sections by placing the slides in amyl acetate with constant agitation for 2h. They were then hydrated through an alcohol series to water. The preparations were placed in 5% TCA at 4°C for 5m, rinsed in water and dehydrated through an alcohol series before being air dried from acetone. They were then coated at 45°C with Kodak NTB 2 liquid emulsion, diluted 1:1 with water, placed in light tight boxes, and left to expose for periods of 20 - 40 days at 18°C. The preparations were developed in Kodak D 19b developer for 3m at 18°C, rinsed in distilled water, fixed in Kodak Metafix for 5m, washed and air dried. The sections were stained for 1 - 2m in a 0.5% solution of methylene blue (G.T. Gurr, London) in 1% (w/w) sodium tetraborate (borax). They were rinsed in distilled water, passed rapidly through an alcohol series to absolute alcohol, cleared in xylene, and mounted in Canada balsam.

In the two hour preparations, only the nucleoli of the nutritive cells were labelled. In 8h preparations, nuclei and cytoplasm of the nutritive cells were labelled. The trophic core and the front ends of the tubes were labelled, but further back, the tubes were unlabelled.

The trophic region of the 12h and 24h preparations were heavily labelled (Fig. 13) and the nutritive tubes were labelled throughout their lengths (Fig. 14). The intensity of labelling over the tubes, however,

decreased progressively towards the posterior ends of the ovarioles. The cytoplasm of the oocytes was labelled above background. The situation was reversed in the three week preparations, in that the cytoplasm of the oocytes was almost twice as heavily labelled as any part of the trophic region or nutritive tubes. The oocyte nuclei were scarcely labelled in any of the preparations (Figs.13,14,15). The follicle cells were labelled in all preparations.

The movement of labelled RNA from the trophic core down the nutritive tubes was monitored by grain counting of the 2h, 8h, 12h, 24h and 3w preparations. Longitudinal sections were taken, and the number of grains inside a $150\mu m^2$ grid were counted above the middle and hindmost regions of the trophic core and above regions down the nutritive tubes alongside the 1st, 2nd, 3rd, 4th, etc., follicles and also above the cytoplasm of the seventh follicle. These counts are given in Table 3 and in histogram form in Fig.17.

The grain counts at different times of exposure to ^{3}H-uridine, can be best explained by the synthesis of RNA within the nutritive cells, the passage of RNA into the trophic core, down the nutritive tubes to the oocytes, and storage within the oocytes. The germinal vesicle synthesised little or no RNA during this stage in oogenesis, while on the other hand, the follicle cells were active in the synthesis of RNA.

In autoradiographs of transverse sections through ovarioles, which had been exposed to ^{3}H-uridine for 12h or more, most of the nutritive tubes were labelled, although a few were quite unlabelled (Fig. 15).

Table 3

Number of silver grains over an area of 150μ^2 in two regions of the trophic core, regions of nutritive tubes alongside successive follicles and cytoplasm of the 7th follicle, in preparations fixed at different times after ^{3}H-uridine injection. (Each number is the mean of four counts, each count being made over a different area). The number '0' indicates that the grain count was not significantly above background.

Fixation time	Trophic core		Label above nutritive tubes opposite follicles						Cyto-plasm of 7th follicle
	Front	Rear	1	2	3	4	5	6	
2h			U N L A B E L L E D						
	12	2	1	2	1	1	1	1	0
8h	18	8	2	3	0	1	0	0	0
	10	7	1	0	0	0	0	0	0
	11	8	2	1	2	2	0	0	0
	20	13	8	9		5			4
12h	19	14	11	8	3	3	7	9	3
	18	14	11	9	7	6	6		5
	20	13	13	10	6	12	9	7	4
	83	58	22	14	17	20	20	16	9
24h	44	39	11	13	12	10	9	7	5
	41	20	12	7		11	10	13	7
	38	24	13	11	11	12	9	7	4
	6	7	6	6	7	9	12	13	18
3w	5	7	7	7	7	9	12	13	21

The question arose as to whether the nutritive cells are the sole source of RNA for the oocytes. Since my autoradiographs showed that there was no accumulation of labelled RNA around the periphery of the oocytes, and since heavily labelled follicle cells could be seen surrounding unlabelled oocytes and nutritive tubes (Fig.16), I conclude that RNA does not enter either the oocytes or the nutritive tubes from the follicle cells. Therefore, the RNA which accumulates in the oocytes during oogenesis must come exclusively from the trophic region of the ovariole.

Since there is a synthesis and transport of RNA, probably ribosomal, from the trophic region to the oocytes, I decided to locate the position of the ribosomal genes within the nutritive cells and see whether they are associated with the large nucleolus, which is a conspicuous feature of these polyploid cells. This can be achieved by '*in situ* cytological hybridisation', a technique developed by Gall and Pardue (1969) which was in use in the cytology laboratory at Leicester at the time of my study. Using this technique, specific nucleotide sequences can be located within cytological preparations by annealing ^{3}H-labelled RNA or DNA to denatured complementary DNA in cytological preparations. The sites of hybridisation are then detected by autoradiography.

The RNA used in this experiment was a highly radioactive RNA complementary to purified ribosomal DNA extracted from ovaries of *Xenopus* a few weeks past metamorphosis. The complementary RNA was prepared by *in vitro* transcription as described by Pardue, Gerbi, Eckhardt and Gall (1970) and was kindly given to me by Dr. J.G. Gall (Yale University, U.S.A.).

In situ cytological hybridisation:

Single trophic regions were cut from ovarioles and placed in 3:1 absolute ethanol:glacial acetic acid. Each was then transferred, with extreme care, to 10µl of 45% acetic acid on a siliconised cover slip. Care was taken not to transfer too much 3:1 fixative. Coverslips were then inverted over 'subbed' glass microscope slides and squashed with varying degrees of pressure. The cover slips were removed by the 'dry-ice' method and the slide plunged into 95% ethanol, removed, and then allowed to dry. Basic proteins were removed from the preparation by placing the slide in 0.2 N HCl at room temperature for 30m, washing it prior to drying the slide in air. The endogenous RNA was removed by placing the slide in a solution of RNase at 37°C for 1h (the RNase solution contained 100µg/ml of RNase in 2 x SSC). Preparations were rinsed twice with 2 x SSC, passed through 70% and 95% ethanol and dried in air. Preparations were then denatured in both acid and alkali. For alkali denaturation, slides were placed into 0.07N NaOH at room temperature for 2m, and for acid denaturation, into 1N HCl for 2m. After denaturation the slides were transferred to 70% ethanol for 10m with at least two changes. This was followed by rinsing in 2 - 3 changes of 95% ethanol for 10m before air drying. For the formation of the hybrid, 50µl of the Xenopus ribosomal complement-^{3}H (specific activity approx. 10^{8}dpm/µg) was pipetted onto the squashed tissue, which was then covered with a coverslip, and the slide placed into a moist chamber containing 2 x SSC to remove the coverslip, washed in the same solution and then placed into RNase solution (20µg/ml in 2 x SSC) for 1h at 37°C.

The slides were coated with liquid emulsion, allowed to expose for periods of 10 - 20 days, developed and then stained with Giemsa, and permanently mounted in Euparal.

Silver grains were evenly scattered over the nuclei of the nutritive cells, regardless of their size (Fig. 18), and were not specifically associated with the heterochromatin or the nucleoli.

c) AN ELECTRON MICROSCOPE STUDY OF THE FINE STRUCTURE OF OVARIOLES.

Because of the movement of RNA from the trophic region down the nutritive tubes to the oocytes, I looked at the ultrastructure of these tracts and also at the trophic core.

Electron microscope:

For electron microscopy, single ovarioles were freshly excised into a 5% solution of glutaraldehyde in 0.06M phosphate buffer at pH 7.3 and 18°C. After 15m in glutaraldehyde, the ovarioles were washed briefly in phosphate buffer and transferred to 1% osmium tetroxide buffered to pH 7.3 with veronal acetate (Palade, 1952). The ovarioles were then dehydrated in acetone and embedded in Vestopal W. Other ovarioles were dehydrated in ethanol, placed in propylene oxide and thence into araldite. The araldite was polymerised in three stages: 12h at 30°C, 12h at 45°C and 12h at 60°C. Silver to grey sections were cut with glass knives on a Cambridge ultramicrotome Mk I & II (A.F. Huxley pattern), mounted on Athene 483 grids without supporting films and doubly stained with uranyl acetate for 5m

and lead citrate for 2m (Reynolds, 1963). With the sections embedded in araldite, the length of staining time with uranyl acetate was increased to 1h. All the staining solutions were filtered through a Millipore filter (0.45μm pore size) immediately before use. Sections were examined with a Siemens Elmiskop 1 and an A.E.I. EM 802 electron microscope at negative magnifications between 4,000 and 60,000.

The electron microscope shows that the trophic region of the ovariole is syncytial (Fig. 19). Trophic nuclei 15μm wide have a large nucleolus which almost fills the nucleus. Amongst the nuclei are scattered membranes and the cytoplasm around the trophic nuclei is rich in mitochondria and packed with particles, which on the basis of their size and general appearance in electron micrographs, I have assumed to be cytoplasmic ribosomes. Amongst the ribosomes are randomly oriented microtubules. The trophic core is packed with a dense mixture of ribosomes and microtubules (Fig. 20). The microtubules in the core are parallel and so numerous that they give the core a streaky appearance along its length, and where trophic nuclei impose on the edge of the core, microtubules form flow patterns around these (Fig. 20).

The nutritive tubes are filled with an extensive system of parallel microtubules which are interspersed with ribosomes (Figs. 22, 23, 24). The density of packing of microtubules appears roughly constant along the length of a nutritive tube. There does not seem to be a distinct pattern of microtubules or ribosomes in the nutritive tubes. However, in longitudinal sections where there are close alignments of microtubules, the cytoplasm between them is relatively free from ribosomes, and ribosomes are clustered between groups of microtubules.

I have estimated that, in a transverse section of a nutritive tube 15μm wide, there are approximately 30,000 microtubules. In transverse sections, microtubules appear as dark staining circles, with an outside diameter of 20nm and a lighter centre (Fig. 25). Around individual microtubules, for a distance of 30nm, there is an electron-opaque zone from which ribosomes are excluded (Fig. 25). In longitudinal sections, lengths of microtubules of up to 2μm can be seen. In longitudinal sections, where there is an inpushing of the wall of the nutritive tubes (Fig. 22) there is an absence of microtubules on either side of the inpushing, and a close packing of tubules at its apex.

The cytoplasm of the oocytes is packed with ribosomes with a few scattered microtubules (Fig. 26). The folded membrane of the oocytes interdigitates with those of the surrounding follicle cells (Fig. 27).

Where microtubules are found in parallel masses, the aggregation is invariably birefringent when examined between crossed polaroids (see Inoué & Bajer, 1961). I therefore examined ovarioles in polarised light.

Examination of ovarioles in polarised light:

Ovaries were removed and placed in Ringer solution. Single ovarioles were dissected out carefully, and freed from as much trachial tissue as possible. They were then mounted in clean Ringer solution on a slide. I found it important to handle the ovarioles by pipette, and as gently as possible, since careless dissection with forceps tended to stretch and displace the nutritive tubes. The ovarioles were then examined between crossed polaroids fitted to a Carl Zeiss photomicroscope POL.

The trophic core showed strong birefringence, with fainter birefringent extensions between the trophic nuclei (Figs. 28, 29). The nutritive tubes, leading backwards from the core, were also strongly birefringent. They passed in a basket fashion around the prefollicular region (Fig. 30), and followed a spiral course backwards around and along the ovariole. The tubes became progressively fewer in number towards the rear (Figs. 31,32), as one would expect from the one tube/one oocyte relationship.

MICROTUBULES

This section of my study is concerned with an investigation of the morphology of the massive system of microtubules within telotrophic ovarioles, and a series of experiments to determine the action of various antimitotic treatments on cytoplasmic microtubules. The telotrophic ovary has proved to be excellent for the study of microtubules, for many reasons. First, because of the quantity of microtubules - there are approximately 6000,000 in the nutritive tubes of each ovariole. Secondly, the microtubules in the nutritive tubes have a distinct orientation, and are associated with the movement of ribosomes within the ovary over distances of up to 1mm. Thirdly, whole nutritive tubes can be isolated from ovarioles.

a) A NEGATIVE STAIN AND FREEZE-ETCH EXAMINATION OF THE MICROTUBULES IN NUTRITIVE TUBES: A SEARCH FOR CONNECTIONS BETWEEN MICROTUBULES AND RIBOSOMES.

There are many reports of microtubules occurring in regions of cytoplasmic flow (Porter & Tilney, 1965; Bikle, Tilney & Porter, 1966; Sabnis & Jacobs, 1967; Holmes & Choppin, 1968; Sjostrand, Frizell & Hasselgren, 1970; Macgregor & Stebbings, 1970). In telotrophic ovaries, RNA passes down the nutritive tubes in the form of ribosomes, along channels packed with microtubules. The general occurrence of microtubules in regions of cytoplasmic movement suggests that their presence is not merely a coincidence, and there is much debate as to whether the microtubules cause, or simply facilitate this movement (See discussion).

I have used the techniques of negative staining and freeze etching to examine the microtubular system of the nutritive tubes in an attempt to answer a number of specific questions, which could provide clues as to the function of these organelles.

1) Are microtubules, as seen in fixed and sectioned material, present in the living cell?
2) Are there any connections between the microtubules and the ribosomes?
3) Is there a particular arrangement of ribosomes around the microtubules?
4) Are the 'clear zones', seen around microtubules in transverse sections, real?

First, I negatively stained homogenates of ovarioles, to look specifically for connections between the microtubules and the ribosomes. This technique has been used extensively to study the ultrastructure of microtubules, both from preparations of flagellae (Grimstone & Klug, 1966;

Behnke & Zelander, 1967) and from disrupted cells (Gall, 1966; Barnicott, 1966; Kieffer et al., 1966). For negative staining, I have followed the method of Gall (1966).

Negative staining

Initially, ovarioles were lightly homogenised in a small amount of insect Ringer, at room temperature, using a hand homogeniser. A Formvar/carbon coated grid was then touched to the homogenate. Without letting the preparation dry, the grid was transferred to the surface of 1% aqueous uranyl acetate for 1 - 2m, rinsed in water and floated on the surface of a 1% sodium phosphotungstate solution (pH 6.6). The excess stain was removed by touching to filter paper and the grid allowed to dry before examination.

Using this technique, which had stained and preserved the microtubules of frog erythrocytes (Gall, 1966), I did not observe any microtubules on the grids. I suspected that the microtubules were labile and had been destroyed by the homogenisation. I therefore searched for a method of stabilising the tubules. I started by using dilute solutions of glutaraldehyde, because it is known to preserve the structure of microtubules.

Ovarioles were immersed for 2 - 3m in buffered glutaraldehyde, and the effectiveness of the treatment estimated by examining pieces of ovarioles using a polarising microscope.

The birefringence of nutritive tubes in short lengths of ovarioles was preserved, and in some preparations, intact birefringent tubes had exuded from the cut ends of the ovarioles. In other cases, ovarioles

which appeared brittle after the treatment with glutaraldehyde had fractured, and lengths of nutritive tube were seen to bridge the gap. All the concentrations of glutaraldehyde which were used preserved the birefringence of the nutritive tubes, even in short lengths of ovariole. However, after such treatment, it was not possible to spread the contents of the tubes onto grids.

I then decided to use a dilute solution of formaldehyde in insect Ringer in the hope that the tubules might be preserved, while still allowing separation and spreading.

> Ovaries were dissected out and placed in 0.001% buffered formaldehyde (diluted from 20% with insect Ringer). After 1½m and 5m, the ovaries were removed, washed in Ringer and then teased out using tungsten needles into a small drop of insect Ringer. Coated grids were touched to the drop, to pick up ovarian material, and stained as described previously.

Using this technique, microtubules could be seen in roughly parallel rows over parts of the grids (Fig. 34). Amongst the tubules were round structures (Figs. 33, 34), which I assume to be ribosomes; because of their size, and because they are the only other visible component of thin sections of nutritive tubes. In these negatively stained preparations, some of the ribosomes were freely distributed between the microtubules, while many were in contact with the tubules. Indeed, in many regions the surfaces of the tubules appeared to be encrusted with ribosomes (Fig. 34) Unfortunately, the substructure of the tubules was not well preserved after this treatment with formaldehyde, and it was not possible to observe any precise connections between the tubules and the ribosomes.

In an attempt to improve the preservation of the fine structure of isolated tubules, I turned my attention towards a technique used by Kane (1962) to stabilise and isolate the mitotic spindle. This technique has been modified recently by Kirkpatrick (1969). Both reports showed that microtubules were preserved using hexylene glycol in conjunction with cold and a slightly acidic pH; and that even extremely labile microtubules were stabilised after this treatment. I employed this technique to examine the contents of the nutritive tubes of Notonecta.

I dissected individual trophic tubes from ovarioles. For this, ovaries were removed and placed in a 5:1 salt solution (0.1 M KCl: 0.1M NaCl). Using very fine sharp forceps, the outer epithelial sheath which surrounds the ovariole was gripped, pulled up over the germarium, and removed altogether, just as the removal of a sock from a foot. This left a string of follicles attached to a germarium by nutritive tubes. Using the forceps, a piece of the follicle wall, in the neck region of the ovariole, was pulled away, and this usually revealed a number of translucent nutritive tubes, which appeared birefringent when examined with a polarising microscope.

A coated EM grid was placed at the bottom of a bored slide chamber filled with buffered hexylene glycol (1M hexylene glycol in 0.003M potassium buffer pH 6.5), and the slide placed on ice. The germarium, with the tubes attached, was transferred to the chamber using a Pasteur pipette, and the tissue allowed to settle onto the surface of the grid. The grid was lifted from the chamber with forceps, and negatively stained.

With this isolation procedure, the microtubules were seen in bunches and were well preserved (Fig. 35). As with the preparations treated with formaldehyde, ribosomes were seen between the microtubules, and sometimes touching them. Where individual tubules were not bounded by ribosomes, that part of the tubule was more collapsed, and in some cases tubules were split open (Fig. 36). In split tubules, 11 parallel sub-filaments were visible in the tubule wall and repeated units of 4nm were measured along each sub-filament. Using the negative staining technique, the 'clear zone' which was visible around individual microtubules in thin sections, was not evident, and ribosomes were in contact with microtubules.

There are a number of reports that where microtubules are found in tracts of cytoplasmic flow, a 'halo' or 'clear zone' has been observed around individual microtubules and that other cellular material is excluded from this zone (Ledbetter & Porter, 1963; Silver & McKinstry, 1967; Macgregor & Stebbings, 1970). It has been suggested that this clear zone may be the result of shrinkage on fixation (Maser & Philpott, 1964), but Lane and Treherne (1970) found that if axons from cockroach ganglia were fixed and stained with lanthanum hydroxide, as opposed to the usual preprative techniques, then the clear zone and the 'core' of the tubules stained alike, suggesting that these are real structures. To investigate the structure of microtubules and the positioning of ribosomes in relation to microtubules in as near to the <u>in vivo</u> condition as possible, I used the freeze-etch technique. With this technique, microtubules which had not been subjected to any chemical fixation could be observed within nutritive tubes.

Freeze etching:

For freeze etching, ovaries were removed, and small pieces of ovarioles placed in 20% glycerol in insect Ringer for 10m. The portions containing the smallest oocytes and the most trophic tubes, the 'neck' regions of the ovarioles, were then excised and mounted on gold collared specimen holders before freezing in Freon 22. The freeze etching method was according to the new standard technique of Moore & Muhlethaler (1963). The etched specimens were shadowed with platinum carbon from an electron beam source in a 'Balzer' 360M freeze etcher. The replicas of the frozen fractured surface were cleaned using commercial bleach (Chloros, I.C.I.) for 3h followed by 70% sulphuric acid for 6h, and then mounted on Formvar coated grids. The replicas were examined using an AEI EM 802 electron microscope.

Examination of frozen etched replicas clearly showed microtubules within the nutritive tubes. In longitudinal fractures, the microtubules were straight, almost parallel and rarely touching each other (Figs.37,39). In longitudinal fractures parts of microtubules had fractured over their surfaces, while in other parts, the fracture had occurred through the 'lumen' of the tubules (Fig. 39). In the latter cases especially repeats of approximately 4nm could be seen along the lengths of the tubules, (Fig. 37), thus corresponding with the negative stained image.

Oblique and transverse fractures of the nutritive tubes showed circular profiles of microtubules randomly positioned within the trophic tubes. These were approx. 26nm in diameter, compared to 20nm diameter of fixed and sectioned microtubules. In oblique fractures parallel sub-filaments could be seen in the walls of the tubules (Fig. 40).

In transverse fractures, small 'cog-like' projections could be seen around the fractured tubules (Fig. 38) and in longitudinal fractures hairy projections can be seen from tubules and more clearly between closely adjacent tubules (Figs. 39,41). These may arise as a result of ice crystals growing between microtubules such that the bridges are merely eutectic structures, while on the other hand they could be real and functionally important.

Transverse and oblique fractures show that the clear zone around the tubules and the lumen of the tubules etched to the same extent.

b) INVESTIGATION OF THE EFFECTS OF ANTIMITOTIC AGENTS ON MICROTUBULES AND THE BINDING OF COLCHICINE AND VINBLASTINE SULPHATE TO MICROTUBULE PROTEIN.

Microtubules have been shown to be destroyed, and hence lose their birefringence, when subjected to cold treatment (Inoué, 1952a; Tilney & Porter, 1967) and to colchicine (Inoué, 1952b; Tilney, 1968). I therefore set out to test the effects of these two agents on the system of microtubules found in the trophic core and nutritive tubes, monitoring the effects of the two treatments by polarising and electron microscopy.

Effects of cold and colchicine on microtubules:

A female Notonecta was kept at 2°C for 12h. It's ovary was removed, and a single ovariole placed in Ringer alongside an ovariole from an animal which had been kept at room temperature. The preparation was then examined in polarised light. Other ovarioles from the animal which had been subjected to cold treatment were fixed for electron microscopy. A similar experiment was performed with colchicine. One ovary from an animal was placed in Ringer and the other ovary was placed in a 1% solution of colchicine in the same Ringer. After 1h, 3h, and 6h, an experimental and a control ovariole were placed side by side on a slide and examined in polarised light. Other ovaries, which had been kept for 3h in Ringer, 0.1%, 1.0% and 2.0% colchicine, were fixed for electron microscopy.

Cold treated ovarioles showed reduced birefringence of the core and the nutritive tubes (Fig. 42), but the nutritive tubes never lost all their birefringence.

Ovarioles which had been treated with colchicine (1.0%) for 1h showed birefringence of the core and nutritive tubes, which, after 3h, was lost altogether. Intact microtubules were seen in electron micrographs of nutritive tubes of ovaries which had been treated for 3h with 0.1% colchicine (Fig. 44), although the preservation of the tissue, after sustained periods in solutions of the drug, was not good. After treatment with 1.0% colchicine, there were fewer microtubules in the nutritive tubes and C-shaped 'tubules' were occasionally seen (Fig. 45). Microtubules were rarely seen in nutritive tubes of ovarioles treated with 2.0% colchicine for 3h, and ribosomes were scattered evenly over the cross sections of the tubes (Fig. 46).

At the time I was investigating the effects of cold and colchicine treatment on the microtubules in ovarioles, there were two reports that an antimitotic drug, called vinblastine sulphate (VBL) destroyed microtubules and induced the formation of birefringent crystals in the cytoplasm of treated cells (Bensch & Malawista, 1968; Malawista & Sato, 1969). Both reports inferred that the crystal structures were formed from the breakdown products of the tubules and that isolation of the crystals would provide pure microtubule material for biochemical analysis.

I decided to investigate the effects of VBL on the microtubules in the telotrophic ovary by both polarising and electron microscopy. In addition to altering the cell structure, there were reports that VBL interfered with the nucleic acid synthesis in cells, and hence ultimately protein synthesis (Creasey & Markiw, 1964; Desjardins, Grogan, Arendell &

Busch, 1967). For this reason I also investigated the effects of the drug on the incorporation of ^{3}H-uridine into ovarioles.

Treatment of ovaries with vinblastine sulphate:

Ovaries were bathed in solutions of vinblastine sulphate ('Velbe', Lilly) (VBL) in Ringer, prior to electron microscopy, and examination in polarised light. Excised ovaries were bathed in 10^{-4}M, 10^{-3}M and 2 x 10^{-3}M VBL and also in Ringer. VBL-treated ovarioles were examined at intervals and compared with ovarioles which had been kept in Ringer. For electron microscopy, whole ovaries were bathed for 6h in 10^{-4}M, 10^{-3}M and 2 x 10^{-3}M VBL in Ringer and then fixed as previously described.

VBL destroys the microtubules in ovarioles, as did cold and colchicine. However, in contrast to cold and colchicine treatments, VBL induces the formation of crystalline structures in the cytoplasm of tissues subjected to the drug (Figs.48, 49). Electron micrographs showed crystalline structures in the nutritive tubes, especially close to the walls of the tubes, and sometimes in contact with them (Fig. 49). The crystals were rare in the oocytes, but did occur in the cytoplasm of the surrounding follicle cells (Fig. 48). In the nutritive tubes, the long axes of the crystals were usually parallel to the tube walls. Crystals ranged in size up to 1μm across and 4μm in length and in cross section had a honeycomb appearance (Fig. 50). Each hexagonal unit had an internal diameter of approximately 18nm with a rim of approximately 6nm. In many cases, tubular sub-units could be seen in the rim. In longitudinal sections, the crystals appeared as dark parallel lines 25 - 30nm apart with darker dots at intervals along their length (Fig.51). Dots on adjacent rows

were not positioned in register, but slightly out of step.

Although the overall effect of VBL was to destroy microtubules and induce the formation of crystals, in many tubes intact microtubules co-existed with crystals (Fig. 51). In some cases too, individual microtubules were surrounded not by ribosomes, as in the controls, but by a loose crystal lattice (Fig. 52).

Treatment with 10^{-3} M VBL destroyed microtubules in the trophic region of ovarioles, and crystals similar in size and sub-unit structure to those in the nutritive tubes were seen in the trophic core, amongst the scattered ribosomes. Randomly orientated crystals were found in the cytoplasm of the nutritive cells. The quantity of ribosomes in the nutritive cells was greatly reduced by VBL, while some of the remaining ribosomes were aligned in rows. These rows of ribosomes were sometimes continuous with the crystals themselves (Fig. 53). There was an increase in the amount of rough endoplasmic reticulum in the nutritive cells and follicle cells after VBL treatment (Fig. 48).

Microtubules were rarely seen in nutritive tubes after 2×10^{-3} M VBL treatment for 6h (Fig. 59). The number of ribosomes was also reduced and the tubes appeared empty in comparison with the controls. Crystals were larger with less well defined boundaries. Open lattice networks with crosslinks of varying lengths cover much of the cross sections of the tubes (Fig. 54), and unlike the compact crystals these networks are not composed of such regular hexagonal units. The long axes of the crystals in the nutritive tubes were parallel to the tube walls while the smaller crystals in the cytoplasm of the follicle cells were randomly oriented.

Treatment with 10^{-4}M VBL for 6h had a relatively slight effect on the number and distribution of microtubules and ribosomes in nutritive tubes. Crystals were evident around the walls of the tubes (Fig. 55), and were usually separated form the tube wall by ribosomes. The sub-units of the crystals were the same size as those produced by other VBL concentrations.

Experiments to examine the effects of VBL on the incorporation of ^{3}H-uridine into RNA of ovarioles were performed as follows.

> One of the two ovaries from an animal was placed in 1ml 10^{-3}M VBL, and the other in 1ml of Ringer solution. The ovaries were bathed in the Ringer and the VBL solutions for periods of 0.5h, 1.0h and 1.75h. 10μl (10μCi) of a solution of uridine generally labelled with tritium (Radiochemical Centre, Amersham, England) (30 Ci/mM), were then added to each and incubated at 20°C for 3h. The ovaries were fixed in ice cold trichloroacetic acid (TCA) for 10m and then washed repeatedly in cold 70% ethanol. Single ovarioles were dried in glass scintillation vials and digested with NCS reagent (Nuclear Chicago). The number of counts per minute were measured using a scintillation counter (Nuclear Chicago), and the disintegrations per minute calculated by comparison with a known external barium standard. The disintegrations per minute (dpm) per ovariole treated with VBL was compared with that of control ovarioles.

Levels of 10^{-3}M VBL produced an 87% inhibition of incorporation of uridine into RNA. As can be seen from Table 4, the inhibition of RNA synthesis decreased with longer exposures to the drug. This suggests that the synthetic process, which is initially inhibited by the drug, may partially recover.

Table 4

Incorporation of ^{3}H-uridine into individual ovarioles over a period of three hours, after different durations of pretreatment with 10^{-3}M VBL. (Each number is the mean count of 7 ovarioles).

Duration of pretreatment with VBL prior to the addition of ^{3}H-uridine	Control ovariole in Ringer (dpm)	Ovariole in 10^{-3}M VBL (dpm)	dpm of VBL treated ovariole as % of control
0.5h	27,491	3,590	13.1%
1.0h	39,203	6,159	15.7%
1.75h	28,695	13,538	47.2%

The overall effect of VBL is to induce the formation of crystals, destroy the microtubules and also deplete the ribosomes. Since, in the long term, VBL does not prevent the synthesis of RNA, the reduction in the quantity of ribosomes in the nutritive tubes is likely to be due to a direct effect of the drug on the ribosomes, and possibly their assembly.

Bensch and Malawista (1969) found that VBL destroyed the majority of microtubules in both L-strain filbroblasts and human leucocytes and that crystals subsequently appeared in the cytoplasm of treated cells. Similarly, VBL induced the formation of crystals in the unfertilised oocytes of the starfish Pisaster ochraceous (Malawista & Sato, 1969). In both reports, the authors suggested that the crystals were formed from microtubules and that isolation of the crystals should provide a means of obtaining relatively pure microtubule protein.

What are the crystals composed of? Are they purely microtubular, as suggested by Bensch and Malawista (1969) and Malawista and Sato (1969) from their ultrastructural studies. Certainly my observations indicated that the crystals could contain a ribosomal component also. I considered that the question of crystal composition could best be determined by in vitro studies. I therefore decided to investigate the effects of VBL on samples of microtubule protein (tubulin), to observe whether VBL induces crystals in vitro, assuming of course that the crystals induced in vivo were formed from the breakdown products of microtubules.

Isolation of colchicine bound protein

Colchicine bound tubulin was isolated as per Borisy and Taylor (1967). Ovaries were dissected from 25 female Notonecta and placed in 2ml SMT (0.24M sucrose 0.01M Tris, pH 7.0 with 10mM $MgCl_2$). The ovaries were homogenised with a glass hand homogeniser and spun at 2,500g for 30m and the pellet discarded. The resulting supernatant was spun at 110,000g for 1h, and the 110,000g supernatant used for this experiment. The supernatant was incubated with ^{3}H-colchicine (Radiochemical Centre, Amersham, England) at 3.7×10^{-4}M and 37°C for 1.5h and then applied to a Sephadex G-100 column with 0.01M phosphate buffer pH 7.0. Sixty-eight 1.5ml fractions were collected. The radioactivity (cpm) and protein concentration (μg/ml) in each fraction were counted and estimated. 0.2ml of each fraction was added to 5ml scintillation fluid in a glass scintillation vial and counted with a liquid scintillation counter (Nuclear Chicago). Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

A graph of radioactivity in the different samples, showed two peaks, similar to those obtained by Borisy and Taylor (1967). The first of these peaks corresponded with samples containing a high protein fraction, while the other much larger peak represented unbound colchicine. The effect of VBL on different fractions was examined.

VBL at a final concentration of 5×10^{-4}M was added to fractions 1 and 2, which contained most of the tubulin, and also to fractions 9 and 10, as controls.

On addition of VBL to fractions 1 and 2, the mixture became cloudy and a heavy white precipitate formed. VBL also produced a precipitate when added to fractions 9 and 10, but to a lesser extent. This indicated that VBL precipitated tubulin, even though the drug colchicine was already bound to the protein. However, it also showed that VBL precipitated other proteins and therefore did not bind specifically to tubulin.

This posed the question: what is the significance of the white precipitate which formed when VBL was added to the tubulin, and what is the correlation, if any, betwwen this precipitate and the crystals induced by VBL *in vivo*? To determine whether the precipitate showed any crystal structure, I fixed a portion of the precipitate for examination by electron microscopy.

> The white precipitate produced on the addition of VBL to the tubulin fractions (fractions 1 and 2) was centrifuged lightly to a pellet, and fixed for electron microscopy with glutaraldehyde and osmium tetroxide as previously described.

Sections of the precipitate showed densely staining amorphous material (Fig. 56) which in places showed circular profiles and configurations which I interpreted as sections of helical structures. No crystalline structures were obvious in any of the sections, which I examined. Ladder-like arrangements have been seen by Bensch, Marantz, Wisniewski & Shelanski (1969) in sections of tubulin precipitated by VBL, and Marantz and Shelanski (1970) have examined similar precipitates by negative staining and described 'bedspring-like' arrays of helices.

To investigate the specificity of binding of VBL to tubulin, I analysed VBL precipitates biochemically. High speed supernatants of ovarian homogenates were treated with VBL, and the resulting precipitates and also the VBL supernatants were run on acrylamide gels.

Acrylamide gel electrophoresis:

Ovaries were dissected from 15 Notonecta into 1ml sucrose tris (ST) and homogenised in a hand homogeniser. During homogenisation, the degree of breakdown of the tissue was examined by phase contrast microscopy. When the majority of the cells had been disrupted, the homogenate was spun at 105,000g for 1.5h.

The supernatant was divided into 0.4ml and 0.5ml portions. VBL was added to the 0.4ml portion to give a final concentration of 2×10^{-3}M and added slowly, so that an interface was formed between the drug and the supernatant. This was left for 30m and a precipitate formed at the interface. The mixture was then spun at 30,000g for 15m. The precipitate was resuspended in reducing solution, and both the resuspended precipitate and the supernatant protein samples reduced and alkylated as per Renaud, Rowe & Gibbons (1968). The amounts of protein in the original 105,000g supernatant, the VBL supernatant and the resuspended VBL precipitate were estimated by the method of Lowry, Rosebrough, Farr & Randall (1951). Electrophoresis was carried out on 7.5% acrylamide gels containing 8M urea. Samples of the 105,000g supernatant, the VBL precipitate and the VBL supernatant were loaded onto different gels. A sample from Chlamydomonas flagellae, kindly

given to me by Dr. J.B. Olmsted (Yale University, U.S.A.) was also run as a control.

The quantities of the different samples, which were loaded onto the gels, are given in Table 5.

Table 5

Gel Nos.	Sample	Amount loaded
1	105,000g supernatant	35 µg
2		70 µg
3	VBL supernatant	40 µg
4		80 µg
5	VBL precipitate	100 µg
6	Flagellae sample	50 µg

3 drops of bromophenol blue were added per litre of top buffer and the samples were run until this tracking dye almost reached the bottom of the tubes (2.5h). The gels were then removed from the tubes and stained with 1% fast green in 7% acetic acid for 2h (Gorovsky, Carlson & Rosenbaum, 1970) and then destained by constant agitation in 7% acetic acid for 2 days.

The distances migrated by the tracking dye and the different bands within the gel were measured, and the mobility (RM) of each band relative to the mobility of the tracking dye calculated.

This allowed the comparison of certain bands in different gels.

The gels were also scanned at 617.5mμ (wavelength of maximum absorbance of fast green) using a Joyce Loebl spectrophotometer.

Examination of the banding patterns in the gels showed that the number of bands resolved was, to some extent, a function of the amount of protein loaded (See also Raff, Greenhouse, Gross & Gross, 1971). There were at least 11 proteins in the VBL precipitate, and approximately 9 in the VBL supernatant. Since some of these bands have the same RM value, it is possible that not all of a particular protein was precipitated by the VBL at the concentration used. Scans of the four gels are shown in Figs. 57 & 58, and the RM values of the bands in each of the gels are given in Table 6. The RM values of the tubulin bands in the flagellae gel were 0.257 and 0.279. Examination of the table of RM values, shows bands both in the VBL supernatant and VBL precipitate gels with approximately these RM values.

This experiment showed that at room temperature, VBL precipitated a number of proteins, probably including tubulin, from supernatants of homogenates of the Notonecta ovaries, (of more specific precipitation of tubulin by VBL at 0°C; Raff, Greenhouse, Gross & Gross, 1971 - see discussion).

Since I completed these experiments, Weisenberg and Timasheff (1970) have found that divalent cations mimic the effect of VBL, in precipitating tubulin from supernatants.

Table 6. Banding patterns of 105,000g supernatant, VBL supernatant, VBL precipitate and flagella sample in gels.

Distance travelled by marker dye	Distance travelled by bands in gel	R.M. of bands	Distance travelled by marker dye	Distance travelled by bands in gel	R.M. of bands
	105,000g supernatant (35µg)			(70µg)	
6.70 cms	0.85 cms	0.127 cms	6.9 cms	0.85 cms	0.123 cms
	1.00	0.149		1.00	0.145
	1.20	0.179		1.25	0.181
	1.50	0.224		1.50	0.217
	1.65	0.246		1.65	0.239
	2.05	0.306		2.05	0.297
	2.45	0.366		2.47	0.358
	3.00	0.448		2.75	0.399
	VBL supernatant (40µg)			(80µg)	
6.40 cms	0.86 cms	0.125 cms	6.2 cms	0.75 cms	0.121 cms
	1.00	0.156		0.95	0.153
	1.20	0.188		1.20	0.144
	1.40	0.219		1.40	0.226
	1.55	0.242		1.60	0.258
	1.60	0.250		1.95	0.315
	2.00	0.313		2.30	0.371
	2.35	0.430		2.90	0.468
	2.95	0.461		3.60	0.581
	VBL precipitate (100 g)			Flagella sample (50 g)	
6.9 cms	0.30 cms	0.043 cms	5.8 cms	0.25 cms	0.043 cms
	0.50	0.072		0.50	0.086
	0.80	0.116		0.60	0.103
	0.95	0.138		1.02	0.176
	1.25	0.181		1.20	0.207
	1.70	0.246		1.49	0.257
	2.00	0.290		1.62	0.279
	2.75	0.399			
	3.05	0.442			
	3.50	0.507			
	3.85	0.558			

Because colchicine binds specifically to tubulin (Borisy & Taylor, 1967) and since VBL precipitates tubulin from supernatants, I decided to investigate their affinities for tubulin and the nature of their binding sites on the tubulin by competition experiments between the two drugs. At the same time, I could also investigate my hypothesis that intact microtubules act as a template, seed, or former for the assembly of VBL-induced crystals. I suspected this, since the crystals always have the same orientation as the microtubules which they replace, and where there is no distinct alignment of microtubules, the crystals are randomly oriented.

Bensch and Malawista (1969), reporting their discovery of VBL-induced crystals in mammalian tissue culture cells, stated briefly that pretreatment with colchicine did not affect the ability of VBL to produce crystals. In this case, the concentration of colchicine was low, and the effect of the colchicine on the microtubules was not reported. More recently, Krishan and Hsu (1971) have demonstrated autoradiographically, at the light microscope level, that protein which binds colchicine is incorporated into VBL-induced crystals in mammalian tissue culture cells. In addition, my in vitro experiments have shown that VBL precipitates protein which already has ^{3}H-colchicine bound to it.

I have used colchicine as a tool for dissembling microtubules, prior to treatment with VBL, to discover whether crystals form, and if so, to investigate what effect the predestruction of the microtubules might have on both the structure of crystals and their distinct orientation. By treating ovarioles with colchicine followed by VBL, prior to examination by electron microscopy, I could investigate cytologically reports that colchicine and VBL have separate binding sites on tubulin and do not compete in any way (Wilson & Friedkin, 1967; Creasey & Chou, 1968).

Ovaries were removed from Notonecta, and bathed for 3h in 0.1% and 1.0% colchicine in Ringer. A further batch of ovaries were treated as described, washed in Ringer, and treated for a further 3h with 10^{-3}M VBL. Single ovarioles were teased out and fixed for electron microscopy.

In electron micrographs of ovaries treated with 0.1% colchicine for 3h, the number and distribution of the microtubules in nutritive tubes was similar to that of untreated tubes. In tubes treated with 1.0% colchicine, the number of microtubules was greatly reduced, and some 'C-shaped tubules' were observed.

Ovarioles which had been pretreated with 0.1% colchicine for 3h, followed by VBL, appeared the same as those treated solely with VBL, with distinct crystals appearing like a 'honeycomb' in transverse sections (Fig. 59). After treatment with 1.0% colchicine and the same concentration of VBL, no microtubules were seen in the nutritive tubes, there were fewer ribosomes, and instead of crystals, the tube contained patches of loose feathery material surrounded by the remaining ribosomes (Figs.60,61). In contrast to distinct crystals, this material had no obvious regular structure when viewed in transverse or longitudinal sections. In electron micrographs, the feathery aggregates appeared lighter than the ribosomes, and in transverse sections, circular profiles of varying diameters could be seen in the material, which in places also showed cross linkings of varying lengths.

If therefore microtubules are intact, then VBL induces the formation of regular hexagonal crystals. However, if the microtubules are destroyed with colchicine, then crystals do not form although there is some aggregation

of crystal-like material.

Although VBL and colchicine bind differently to tubulin, both are antimitotic agents which destroy microtubules. My observations show that when the microtubules in nutritive tubes are destroyed by colchicine, the ribosomes which are the only other visible cell component, spread uniformly and fill the gaps left by the tubules. In contrast, after treatment with VBL arcs and circles of ribosomes are seen, and ribosomes do not invade a region previously occupied by a microtubule. This indicates that although VBL and colchicine both destroy the tubule structure which is seen after conventional fixation and staining techniques, they act differently on the surrounding sleeve of electron opaque material and therefore the complete structure of microtubules.

IN VITRO MAINTENANCE TECHNIQUE FOR TELOTROPHIC OVARIES.

The remainder of this work has involved the development of a technique for the maintenance of telotrophic ovaries *in vitro*. To fully understand the action of antimitotic drugs on microtubules, I consider it important to determine whether tubules can reform in tissues after removal of such drugs. An investigation of this kind is not possible *in vivo*, since injected drugs cannot be flushed from live animals, and an *in vitro* maintenance technique is therefore essential.

Attempts to maintain and grow organs of insects *in vitro* have met with varying success. The importance of insect hormones for successful culturing has been reviewed recently by Marks (1970). In some cases ovaries have been found to develop *in vitro* without the addition of any external source of hormone (Stern, 1940; Demal, 1961; Lender & Duveau-Hagege, 1963; Leloup, 1964). However, Lender and Lavadure (1967), on culturing ovaries of *Tenebrio*, and Ittycheriah and Stephanos (1968) investigating the ovaries of *Iphita* found that fat body extract was essential for normal development.

For this study I required to maintain telotrophic ovaries in as life-like a condition as possible - the most important factor being the preservation of the microtubules and the maintenance of ribosomal movement within the nutritive tubes.

In vitro maintenance of telotrophic ovaries:

Ovaries were removed under sterile insect Ringer, washed three times in sterile Ringer, and finally in Grace's Modified Insect Medium (Grand Island Biological Company). The culture chambers used were 5.5cms diameter sterile plastic petri dishes (Nunclon,

Sterilin Ltd.), the bases of which were scratched with a scalpel blade. By scratching at right angles, points of plastic were raised, and these acted as rafts which elevated the ovaries from the bases of the dishes, thus allowing free circulation of medium around the ovaries. The roughened bases also served to anchor the ovaries during the changing of media. The excised and washed ovaries were transferred to the rafts in the petri dishes, which contained 2.5mls of insect medium to which had been added Crystamycin (0.16 mg/ml, Glaxo) and Achromycin (0.02 mg/ml, Lederle). For some of the ultrastructural studies ovaries were also maintained in medium without antibiotics. During long periods of culture, the medium in each dish was changed routinely every twelve hours. All procedures were performed in a portable sterile workbench (Microflow).

The cultured ovaries were examined during culture periods of 48h, in polarised light and by electron microscopy, as described in a previous section.

The trophic cores and the nutritive tubes of the ovaries were strongly birefringent throughout the culture periods of 48h, thus indicating microtubule preservation. Such suppositions were supported by electron micrographs of nutritive tubes, after 48h in culture, which were indistinguishable from micrographs of freshly excised material.

The system whereby ribosomes are transported from the trophic region to the oocytes, *in vivo*, has been described in a previous section. However, since it has been reported that the salivary glands of *Drosophila* failed to synthesise ribosomal RNA (rRNA) *in vitro* (Greenberg, 1969), I considered

it wise to determine for my culture system a) whether RNA is continually synthesised, b) if rRNA is made, and c) whether it is transported down the nutritive tubes to the oocytes.

To clarify the first point, ovaries were cultured as described in a medium containing 50µl/ml ^{3}H-uridine (5Ci/mM: Radiochemical Centre, Amersham). Ovarioles were removed after 2h, 6h, 12h, 24h and 48h, fixed for 5m in ice cold TCA, digested with NCS base (Nuclear Chicago)(0.4mls NCS : 0.03mls water/ovariole), and scintillation fluid added to the vials. The amount of uridine incorporated per ovariole was determined by liquid scintillation counting.

The cpm/ovariole over a 48h period are shown in Table 7, and graphically in Fig. 62. Each number is the mean count from 8 ovarioles. This experiment showed that ovarioles incorporate ^{3}H-uridine linearly into RNA during a 48h culture period.

TABLE 7.

Table of incorporation of ^{3}H-uridine into individual ovarioles. (Each number is the mean count of 8 ovarioles).

2h	6h	12h	24h	48h
3,484 cpm	13,761	16,671	23,933	39,197

To determine whether rRNA is made *in vitro*, ovaries from 5 animals were set up in culture and maintained for 24h. Control ovaries were then removed from a further 5 animals and both sets of ovaries (cultured experimentals and freshly removed controls) were cultured for 6h in a medium containing ^{3}H-uridine (100μl/ml : 30Ci/mM). RNA was then extracted from the experimentals and the controls by the method of Brown and Littna (1964). Ovaries were removed from the rafts and washed in 0.1M NaAc pH 5.0 with 5μg/ml poly-vinyl sulphate (PVS). All extraction procedures were performed cold. The ovaries were homogenised lightly in 5ml of the same buffer and 0.5% sodium dodecyl sarcosinate (Sarkosyl, Ciba-Geigy) added to disrupt the cells. An equal volume of phenol, saturated with water, was added and the tube shaken vigorously prior to centrifugation at 12,000g for 20m in an angled rotor. The upper layer, containing the RNA, was pipetted off, $2\frac{1}{2}$ times the volume of absolute ethanol added and the RNA allowed to precipitate overnight in a freezer. The RNA was collected by centrifugation at 16,000g for 30m. The two samples were washed carefully with 70% ethanol to remove any traces of phenol, and centrifuged again at 16,000g to precipitate. The samples were then resuspended in 2.0ml 0.1M NaAc pH 5.0 with 5μg/ml PVS. The optical density (OD) of the samples was read at 260mμ to determine the RNA content, and 50μl of each sample was spotted onto Millipore filters, and counted by liquid scintillation. 1ml samples were loaded cold onto sucrose gradients (5 - 20%) and spun in a 3 x 20ml swinging bucket rotor on an M.S.E. "Superspeed 50" ultracentrifuge at

22,000K for 20.5h at 10°C. '32 drop' fractions were collected and the OD and cpm of each fraction determined. Both the OD and cpm of the experimental and control fractions were plotted and compared.

The RNA from both the cultured and freshly excised ovaries sedimented in three major bands in sucrose gradients (Fig. 63). The RNA profiles were similar to those of the ovarian RNA of some amphibians (Brown & Littna, 1964; Gall, 1966). The two larger bands are ribosomal RNA, probably with sedimentation coefficients of 28S and 18S although the values of the coefficients vary somewhat in insects and have been found to be 24S and 17S in Oncopeltus fasciateus, another hemipteran (Harris & Forrest, 1967). The smallest peak is the 4S transfer RNA. The sedimentation pattern of the incorporated ^{3}H-uridine, shown in the figures, reveals newly synthesized rRNA in the cultured ovaries. After this short period of ^{3}H-uridine incorporation, the two major peaks of radioactivity ran slightly ahead of the OD profiles.

Finally, to investigate the movement of RNA in vitro, ovaries from three animals were set up in culture and allowed to 'acclimate' for 24h. The ovaries were then transferred to fresh medium containing ^{3}H-uridine (10 l/ml : 5Ci/mM). After incubation times of 3h, 12h and 24h, the ovaries were fixed in Sanfelice fixative, washed in running water for 24h, dehydrated in ethanol and embedded in methacrylate. Sections (0.5µm) were cut with glass knives on a Huxley Ultramicrotome Mk II and mounted on slides. The slides were coated with liquid emulsion for autoradiography, as described previously.

Examination of the autoradiographs showed a progressive movement of labelled RNA with time from the nutritive cells, into the trophic core and thence down the nutritive tubes to the oocytes, as has been described in vivo.

DISCUSSION

Oocytes store RNA, and most of this RNA is ribosomal (Brown, 1964; Brown & Dawid, 1968). Oocyte nuclei have the 4C amount of chromosomal DNA, but in many amphibians and some insects, a high rate of synthesis of ribosomal RNA is achieved by a selective amplification of the ribosomal or nucleolar genes. In such cases, the amplification is evident cytologically as extra chromosomal DNA, produced during or before meiosis (Gall, 1969) and Macgregor (1968) has shown how, in *Xenopus*, this extra DNA is expressed cytologically in the multinucleolate condition of the germinal vesicle. Such an amplification of the ribosomal gene exists in the germinal vesicles of the insect *Acheta* (Lima de Faria, Nilsson, Cave, Puga & Jaworska, 1968) which has a panoistic ovary; and a similar situation exists in *Dytiscus* (Gall, Macgregor & Kidston, 1969), which has a meroistic polytrophic ovary.

In contrast, my Feulgen sections and squash preparations show that the oocytes of *Notonecta* have no extra DNA, and in sections stained with gallocyanine, and electron micrographs, only a few nucleoli are visible in each oocyte nucleus. Despite this, the electron micrographs also show that the cytoplasm of the oocytes is packed with ribosomes. Where do these ribosomes come from?

In *Notonecta*, the oocytes are in direct cytoplasmic communication with the trophic core, via the nutritive tubes. The nuclei of the nutritive cells are packed around the trophic core and sections stained with gallocyanine show that these nuclei have large nucleoli. The fact that the regions around these nuclei and the trophic core are packed with ribosomes, suggests that the nutritive cells make these ribosomes.

Using autoradiography, I have established that there is a progressive movement of RNA from the trophic region to the oocytes, as was suggested by Bonhag (1955). In autoradiographs of ovarioles fixed two hours after the injection of ^{3}H-uridine, there was labelling over the trophic nuclei. In the 8h, 12h and 24h preparations, the labelled RNA had moved into the trophic core and down the nutritive tubes, while after three weeks, most of the labelled RNA had accumulated in the oocytes.

From these observations, it is plain that, in Notonecta, the nutritive cells are an important source of ribosomes for the oocytes. Further, it is evident from examination of Feulgen-stained sections and squashes of the trophic region that the trophic nuclei differ greatly in size and DNA content, and microphotometric measurements showed that the largest trophic nuclei contained at least 140 times as much DNA as the smallest. Fractionation of the DNA of the trophic region of Notonecta on cesium chloride gradients revealed that the DNA formed a single band, and that high density DNA was not present in detectable amounts. I conclude, therefore, that the differences in DNA content of the trophic nuclei is due solely to polyploidy. Indeed, polyploidisation of the chromosomes of nutritive cells of Drosophila melanogaster was observed as early as 1939 by Painter and Reindorp (1939), who estimated that the cells attained 512 ploid.

The structure of the largest trophic nuclei is particularly uniform. In each nucleus there are invariably two large Feulgen-positive blobs of DNA. In heavily squashed preparations, stained with Feulgen, loops of varying sizes can be seen emerging from these heterochromatic masses. A similar finding has been observed in the nutritive cells of both Drosophila melanogaster (Painter, 1940) and Rhynchosciara angelae (Basile, 1969) and the latter author pointed out that these loops resemble those seen on lampbrush chromosomes.

In all likelihood, the loops represent parts of polytene chromosomes. Following an increase in ploidy, polytene chromosomes have been reported in the nutritive cells of some Diptera. They have been seen in the nutritive cells of Drosophila melanogaster (Painter, 1940; Hsu & Hansen, 1953), Lonchoptera dubia (Stalker, 1954) and Calliphora erytrocephala (Bier, 1957). Bier (1957, 1959) showed that polytene chromosomes eventually fuse, leaving only heterochromatic masses and thin filaments, while Schrader and Leuchtenberger (1952) have described a 'laking' or running together of Feulgen-positive material, in the hindermost nutritive cells of the Hemipteran, Acanthocephala bicoloripes. Microspectrophotometric measurements show that the two heterochromatic masses, in the largest nutritive cells of Notonecta, represent approximately 8% of the total nuclear DNA. What is this fraction of DNA? Hybridisation experiments, using the ribosomal complement of Xenopus, indicate that the heterochromatin does not include ribosomal DNA. This is as might be expected, since these cells are highly active in the synthesis of ribosomal RNA and the templates for such synthesis are unlikely to be 'looked up' in heterochromatin. The nature of this fraction of the DNA may be resolved with further cytological hybridisation experiments.

Other materials have been reported to pass from the trophic region to the oocytes in telotrophic ovaries of insects. According to Schrader and Leuchtenberger (1952), in the coreid Hemipteran Acanthocephala bicoloripes, nuclear DNA was exuded as droplets through the nuclear membrane of the nutritive cells, and they assumed that the DNA passed to the oocytes as Feulgen-negative depolymerised substances. Bonhag (1955) reported a similar phenomenon in the ovarioles of the large milkweed bug

and also saw free DNA droplets accumulating near the anterior ends of the 'cords' that lead to the oocytes. Feulgen-stained sections and squashes of ovarioles of Notonecta showed that amongst the trophic nuclei, there are a few which have dense Feulgen-positive droplets and no nucleoli. Autoradiographs showed that these particular trophic nuclei do not synthesise RNA and I suggest that they are 'spent trophic nuclei', the fate of which I therefore consider to be relatively inconsequential.

Bhandari and Nath (1930) stated that the nuclear contents of the nutritive tubes pass along the 'nutritive roots' in the form of a liquid, while Golgi elements pass along intact. They further state that the broken down substances reappear at the distal end of the 'roots' in the form of thick hyaline, gelatinous, and slightly osmiophilic fibres, which they called 'nutritive fibres'. They were not able to stain these fibres, nor isolate them from ruptured eggs. Although they were puzzled as to the nature of the fibres, they suggested that they were the most important food material flowing down the 'roots' from the nutritive cells. I would speculate that the fibres, which Bhandari and Nath described, were either bunches of microtubules, or tracts of ribosomal material lined up by microtubules in the nutritive tubes. I do not think that Golgi material could pass from the nutritive cells to the oocytes and I suggest that the Golgi formations referred to were, once again, mainly attributable to microtubules and ribosomes.

I have estimated that there are about 20,000 nutritive cells and 20 oocytes in each ovariole. The ratio of polyploid nutritive cells to oocytes is therefore 1000 : 1. If the average level of ploidy in the trophic nuclei is 256, then each trophic nucleus may be said to provide 256 nucleolar organisers, and each oocyte may therefore depend upon a supply of ribosomal RNA from 256,000 nucleolar organisers.

Brown and Dawid (1968) have shown that in amphibian oogenesis, the nucleolar or ribosomal genes are always amplified roughly to the same extent, regardless of the size of the nucleolar organiser in a particular species. As already outlined, amplification of the ribosomal genes is found within the oocytes of some insects, but the extent of this amplification is not yet known. Once estimated, however, it will be interesting to compare the number of nucleolar organisers within the oocytes of species where amplification of these genes has taken place, with the number of organisers relied upon by each oocyte, by way of polyploid nutritive cells, in species where no such amplification occurs.

Improvements in preparative techniques for electron microscopy, especially the introduction of glutaraldehyde as a pre-fixative, have enabled the observation of some cellular organelles which were not preserved with other fixatives. One such organelle is the microtubule.

In 1963, Slautterback described small filaments with a central region of low density in the cytoplasm of the ectodermal cells of the coelenterate Hydra, and because of their appearance he named these microtubules. Since then, these organelles have been observed in a wide variety of animal and plant cells, and there are a few reports of intranuclear microtubules (Behnke & Forer, 1966; Tucker, 1967). Understandably, on re-examining old electron micrographs, investigators have been able to identify badly preserved microtubules in tissue fixed solely with osmium.

Many of the reports of microtubules have been from protozoa, where there are descriptions of complex configurations of microtubules forming complete organelles. For instance, the axopods of Heliozoans are composed of aligned microtubules (Tilney & Byers, 1969), as are the cytopharyngeal baskets of ciliates (Tucker, 1968). Microtubules occur in a 9 + 2 pattern in cilia and flagellae (Afzelius, 1959; Gibbons & Grimstone, 1960) and are also the only visible component of the mitotic spindle (Roth & Daniels, 1962; Gonatas & Robbins, 1964; Bajer, 1968).

Until recently, all microtubules were considered to be homologous, due mainly to their morphological similarities. The diameters of tubules varies in different reports between 18 - 23nm, but this can probably be attributed to differences in measuring techniques. However, experiments have shown that microtubules in different situations have different stabilities (Burton, 1968; Behnke, 1970). Indeed, Behnke and Forer (1967) have evidence for four classes of microtubules within individual cells, each class having a different stability. In addition, Olmsted, Whitman, Carlson and Rosenbaum (1971) have found that the two tubules in the doublets of flagellae of Chlamydomonas are differentially susceptible to solubilisation with a mild detergent. Biochemical analysis supports the supposition that not all microtubules are composed of the same material.

The microtubules in the trophic tubes of Notonecta have a similar appearance to those first described by Slautterback (1963). The packed microtubules are oriented parallel to the long axes of the tubes, and are surrounded by ribosomes; but it is rare that ribosomes make contact with the tubules. This gives the impression that individual microtubules are surrounded by a 'halo' of electron opaque material into which the ribosomes do not encroach. A similar 'halo' has been noted around tubules in other cells (Ledbetter & Porter, 1963; Maser & Philpott, 1964; Silver & McKinstry, 1967).

There has been much speculation as to the nature of the 'halo' or 'clear zone', and it has been suggested that this region may be the result of shrinkage on fixation (Maser & Philpott, 1964). However, Lane and Treherne (1970) found that if the axons from cockroach ganglia were fixed and stained with lanthanum hydroxide, instead of the usual preparative technique, then the 'clear zones' around the tubules stained, as did their cores. They concluded that the 'clear zones' were real structures, probably composed of mucopolysaccharide. Substantially similar structural features are seen in transverse and oblique fractures of microtubules, in that the walls of the tubules are dark and the regions around individual tubules, and their cores appear light in shadowed replicas.

Although the freeze-etch technique does not provide any evidence as to the composition of the 'clear zone', my observations indicate that it is a real structure, and not an artifact of fixation. Certainly some authors have stressed that microtubules may have structural components not shown by conventional fixation and staining techniques (Silver & McKinstry, 1967; Forer, 1969).

I have employed the freeze-etch and negative staining techniques so that I could compare the appearance of microtubules in frozen fractured replicas of trophic tubes, with that of microtubules which had been chemically fixed. Certain differences were immediately apparent. In longitudinal sections of nutritive tubes, the microtubules were finely wavy, whereas in both the freeze-etched replicas and the negatively stained preparations, the microtubules appear straight or only gently curved. I regard the straightness to be closer to the true situation, since it seems less likely that wavy microtubules would straighten than vice-versa. Jensen and Bajer (1969) have attributed a corresponding waviness of spindle microtubules in the dividing endosperm cells of Haemanthus, to a post mortem

shrinkage of adjacent long chromosomes.

The information regarding the substructure of microtubules which was obtained from the examination of freeze-etched replicas, corresponds in many respects with that gained from studies in which the microtubules had been subjected to chemical fixation. Sub-filaments have been observed in the walls of microtubules after thin sectioning (Ledbetter & Porter, 1963) and after negative staining (André & Thiery, 1963; Kieffer, Sakai, Solari & Mazia, 1966; Barnicott, 1966). Close examination of my oblique fractures reveals sub-filaments in a sector of the wall of many microtubules. There has been speculation as to the number of sub-filaments within the walls of microtubules, but the freeze-etch technique did not resolve this question, since the sub-filaments were never visible all the way round a single microtubule. Ledbetter and Porter (1964) have used the photographic rotational reinforcement technique of Markham, Frey and Hills (1963) to reinforce the image of microtubules in thin transverse sections, and concluded that there were probably 13 sub-filaments within the walls of the plant microtubules which they examined. Numerous studies of negatively stained microtubules have reported different numbers of sub-filaments in the walls, varying from 10 - 14 (see Behnke & Zelander, 1967) and it is quite possible that microtubules in different situations are composed of different numbers. In my negatively stained preparations, where individual microtubules had split open, and presumably unfolded onto the grid, 11 parallel sub-filaments could be counted.

Using the freeze-etch technique, Northcote and Lewis (1968) have observed microtubules beneath the plasmalemma at the 'cytoplasmic surface' of pea root tip cells and recorded that the tubules showed a distinct structure along their length, the nature of which was not discernible with

the resolution they were able to achieve. Using the same technique to examine yeast cells, Moor (1967) has proposed a microtubule model with the walls consisting of 4nm sub-units arranged in a double helix, with the sub-units aligned in parallel rows corresponding to the beaded sub-filaments seen after negative staining. Freeze-etched replicas of trophic tubes also showed a periodicity of 4nm along fractured microtubules, especially where the fracture had occurred through the 'cores' of the tubules.

Although all microtubules appear morphologically similar, there is considerable evidence that they are not necessarily homogeneous. Apart from their differences in stability, which have already been discussed, biochemical studies have shown that tubules in different situations may have different compositions. Tamura (1971) has isolated different proteins from different microtubular organelles in the protozoan Tetrahymena pyriformis and Olmsted, Witman, Carlson and Rosenbaum (1971) using sophisticated gel electrophoretic techniques, have separated different proteins from the doublet microtubules of the flagellae of Chlamydomonas, and shown that each tubule is composed of more than one protein. In addition, it has been shown that the protein from microtubules is different to that from neurofilaments, both of which were isolated from the axons of squid (Huneeus & Davison, 1970; Davison & Huneeus, 1970).

In contrast, some evidence links microtubules with microfilaments and raises the possibility that they are interconvertible. Microfilaments have been equated with the sub-filaments in the walls of microtubules because they are a similar size and also because masses of microfilaments have been found in the cytoplasm of cells, recovering from the effects of the anti-mitotic drug vinblastine sulphate, where microtubules were present originally (Krishan, 1968).

As the information regarding the composition of microtubules increases, there is gathering evidence as to the way in which they are formed. Microtubules may be many microns in length, and are certainly longer than can often be seen in longitudinal sections. In the nutritive tubes, where there were inpushings into the tubes, microtubules were sparse at the edges of the inpushings, but compact at their apices, which suggests that the tubules were longer than the short lengths which were visible. Possibly individual microtubules extend the lengths of the nutritive tubes. Certainly in some microtubular organelles of protozoans, the tubules run the whole length of the structure (Tucker, 1968).

The question arises, at what point or points along their lengths do microtubules grow? In a series of ingenious experiments, Rosenbaum and Carlson (1969) showed that the flagellae of Chlamydomonas 'grew' from basal bodies by a polymerisation of tubule sub-units, which attach to the ends of each tubule. They have also shown that there is a limited 'pool' of sub-units within the cell body of the animal. Tucker (1970a) has also suggested that there is a polymerisation of tubule sub-units onto the growing end of cytopharyngeal baskets of ciliates, and suggests that the sub-units are the electron-dense material visible in electron micrographs, at the ends of the baskets. Stephens (1969) has reported a similar observation in an in vitro system, where tubule sub-units are assembled on a 'seed' of microtubule which was introduced into the solution of sub-units.

In a telotrophic ovary the nutritive tubes elongate as the oocytes increase in size and progress down the ovariole. This lengthening of the nutritive tubes must necessitate the assembly of microtubular material, since the nutritive tubes are packed with microtubules, regardless of their length. At present I have no evidence regarding the growth of microtubules within the nutritive tubes. However, it is unlikely that microtubules

grow at all points along their lengths, as the diameter of the tubules is relatively constant; and because the nutritive tubes do not contain structures such as centrioles or basal bodies, on which microtubules are known to assemble (Nicklas, 1970), the tubules could grow at either end, or both ends.

I have observed C-shaped tubules in nutritive tubes after treatment with the antimitotic drug, colchicine; and Behnke (1967) has described similar 'tubules' in cells recovering from treatment with cold. However, I do not think this reflects on the normal mode of growth of microtubules.

Two main functions have been attributed to microtubules. They are generally thought to be skeletal, involved in the maintenance of cell assymetry, and to be associated with the movement of cells and within cells (see review, Porter, 1966).

Microtubules are invariably found in outpushings of cells and are a general feature of assymetrical cells, where they are usually aligned parallel to the axis of assymetry. For example, they are found in neural plate cells (Waddington & Perry, 1966; Handel & Roth, 1971), nerve processes (Goldman,1970; Yamada, Spooner & Wessells, 1970), axopods of heliozoans (Tilney & Porter,1965) and the cytoplasmic processes of pigment cells (Bikle, Tilney & Porter,1966). In some of these cases, the microtubules have been destroyed with antimitotic drugs and the result has been invariably a loss of cell assymetry.

The microtubules in the nutritive tubes of telotrophic ovaries may have a skeletal role, in that their presence prevents the collapse of the tubes and thus maintains a passage for the migrating ribosomes. In addition, it is possible that, due to their alignment and close proximity, the microtubules act as a sieve which restricts other cytoplasmic components, such as mitochondria, from entering and clogging the tubes.

The microtubules are probably also involved in the movement of the ribosomes along the nutritive tubes. There are numerous other examples of microtubules occurring in channels of cytoplasmic flow. Bikle, Tilney & Porter (1966) found microtubules in the processes of melanophores, along which the pigment granules migrate in both directions, and suggested that there may be two classes of microtubules in each tract, since the granules migrate in both directions. Tilney and Porter (1965) observed the migration of particles along the complex of microtubules in the axopodia of Heliozoans. Other reports include microtubules in the streaming tracts of the coenocytic marine alga Caulerpa prolifera (Sabnis & Jacobs, 1967) and many descriptions of tubules in the axonal flow regions of neurones of different animals (Kreutzberg, 1969; Fiet, Dutton, Barondes & Shelanski, 1971; Fernandez, Burton & Samson, 1971; Sjostrand, Frizell & Hasselgren, 1970). In some of these studies, where the nervous tissue was treated with the drug, colchicine, the microtubules within the axons were destroyed, and there was a blockage of axonal flow (Kreutzberg, 1969; Sjostrand, Frizell & Hasselgren, 1970).

Holmes and Choppin (1968) found that when a syncitium was induced from cultured cells by the action of a virus, the nuclei migrated to the centre along tracts of microtubules and they likewise found that treatment with colchicine halted the movement of the nuclei.

Syncytia

These examples show that there is a relationship between microtubules and movement, and evidence from those systems treated with colchicine, that the microtubules are essential for movement. However, the question still remains, do the microtubules cause or merely facilitate the movement? This question is difficult to answer, and necessitates a search for an actual method of movement involving microtubules.

Freed and Lebowitz (1970) have found saltatory movements associated with microtubules in cultured cells and proposed that movement of the microtubules could squeeze the cytoplasm along their tracts, much as fingers around a tube of toothpaste.

The main reason for my looking closely at the ultrastructure of the components of the trophic tubes was to look for possible outbranchings or connections between the microtubules and the ribosomes. Microtubule to microtubule cross connections have been reported in a number of situations. They are seen in the axonemes of Heliozoans (Tilney & Byers, 1969; Roth, Pihlaja & Shigenaka, 1970). They connect some of the microtubules in the cytopharyngeal baskets of ciliates (Tucker, 1968) and the axostyles of flagellates (Grimstone & Cleveland, 1965). Where this is the case, it is generally accepted that the cross-bridges function to hold the tubules together, thus providing rigidity, and there is some debate as to whether the cross connections specify the arrangement of the tubules within the complex (Tilney & Byers, 1969; Roth, Pihlaja & Shigenaka, 1970; Tucker, 1970b). There are a few reports of arms or cross-bridges which seem to link microtubules to particles which move alongside them. Pickett-Heaps and Northcote (1966) have noted connections linking vesicles of the smooth endoplasmic reticulum to spindle tubules in wheat meristem cells, and Smith (1971) has described bridges which join synaptic vesicles to neurotubules in the axons of lampreys. Tucker (1972) has observed rows of arm-

bearing microtubules lining the sucking mouthparts of ciliates and has suggested that the arms are involved in the propulsion of materials alongside the tubules.

Smith, Jarlfors and Beranek (1970) propose that the cross-bridges between neurotubules and synaptic vesicles could be a clue to the mechanism of movement of the vesicles. They tentatively suggest that their observations support a hypothesis of Schmitt and Samson (1969) which proposes that a microtubule-particulate association is functionally and perhaps biochemically comparable with the myosin-actin interaction of microfilaments and may be responsible for moving certain components along the axoplasm.

I was not able to detect any cross connections or attachments to microtubules in my negatively stained preparations. This could be due to the inadequacies of the isolation and staining procedures, since there is evidence that, in cilia and flagellae, there are changes in the microtubules following negative staining with phosphotungstinic acid (Henley, 1970). On the other hand, radial links are clearly visible from negatively stained doublet tubules of sperm flagellae (Warner, 1970).

The bridge-like structures between the microtubules, and also the projections from individual tubules seen in my freeze-etched replicas, may be functionally important, while on the other hand, they may be simply eutectic structures, arising as a result of ice crystals growing between the microtubules.

The striking feature of the association between microtubules and cytoplasmic movement is the considerable difference in the nature and size of the organelles which move. For example, ribosomes of approximately 10nm pass along nutritive tubes in telotrophic ovaries, melanin granules of 0.5μm move along the processes of the melanophores of the fish

Fundulus (Bikle, Tilney & Porter, 1966) and in virus induced syncitia, nuclei of 10μm travel along tracts of microtubules (Holmes & Choppin, 1968).

Because of the lack of any obvious unifying feature between these structures which move along tracts of microtubules, I consider it likely that there is a fundamental mechanism of movement which is independent of any property of the moving particle. For instance, the particles, which move, may simply attach to the 'sleeve' or 'clear zone' which exists around each tubule and this sleeve may move down the tubule, possibly ratcheting itself along in a way comparable to the actomyosin interaction of striated muscle. Certainly, actin-like proteins have been found in the neurotubules of squid (Davison & Huneeus, 1970) and in the tubules of cilia and flagellae (Renaud, Rowe & Gibbons, 1968; Shelanski & Taylor, 1968; Stephens, 1968).

The arrangement of microtubules in the trophic core and nutritive tubes render these structures birefringent, as has been seen with mitotic spindles (Nicklas, 1970). Although there is a correlation between the presence of microtubules and the observation of birefringence, it is not known what contribution the tubules make to the total birefringence. Forer (1969) has urged caution in the attribution of birefringence to microtubules alone and Goldman and Rebhun (1969) investigating the structure and properties of the isolated mitotic apparatus, also warn against too strict a correlation, since considerable birefringence remained once the spindle microtubules had been destroyed. They suggest that the 'residual birefringence' may be due to the alignment of the surrounding matrix material.

It has been known for some time that cold treatment (Inoué, 1952a; Goode, 1967) and the drug colchicine (Inoué, 1952b; Behnke, 1965; Brinkley, Stubblefield & Hsu, 1967) arrest mitosis at the metaphase stage of cell division by destroying the mitotic spindle, and both treatments have been widely used in the examination of chromosomes, which are most easily studied at this stage. Since the morphology of the microtubules of the mitotic spindle is similar to that of other cytoplasmic microtubules, I decided to determine whether treatment of the ovarioles of Notonecta with cold and colchicine destroyed the tubules in the nutritive tubes.

Increasing concentrations of colchicine had greater destructive effects on the birefringence of ovarioles, and cold treatment reduced the birefringence, although it was never eliminated altogether by cold. Electron micrographs of ovarioles treated with colchicine showed that the drug destroyed the tubules.

The concentrations of colchicine required to destroy microtubules in different situations varies enormously, but this could be due to differences in permeability of the surrounding cytoplasm, rather than a difference in the stability of different tubules to the drug. In ovarioles treated with colchicine, the birefringence disappears first from the trophic region, and later from the trophic tubes. In this instance, rather than reflecting a difference between the microtubules in the two regions, I think it more likely to be a consequence of the relative impermeability of the walls of the nutritive tubes to colchicine.

A number of treatments other than cold and colchicine have been found to destroy microtubules. Kennedy & Zimmerman (1970) have found that hydrostatic pressure destroys the microtubules of Tetrahymena pyriformis.

Microtubules in the axopodia of Heliozoans have also been found to succumb to pressure (Tilney, Hiramoto & Marsland, 1966), as well as treatment with urea (Shigenaka, Roth & Pihlaja, 1971) and inhalational anaesthetics (Allison, Hulands, Nunn, Kitching & MacDonald, 1970).

Most of the interest and importance attached to the investigation of the effects of antimitotic agents on microtubules stems from the fact that, apart from supplying clues as to the homology of different microtubules, they are proving invaluable tools for research into the mode of assembly, the chemistry and the function of microtubules.

At the time I started to investigated the effects of antimitotic agents on the microtubules in the ovarioles of Notonecta, there were at least two reports of an antimitotic agent, vinblastine sulphate, which, in addition to destroying microtubules, induced crystals in the cytoplasm of treated cells. These crystals had been found in the cytoplasm of mammalian cells (Bensch & Malawista, 1968 & 1969) and also in the oocytes of starfish (Malawista & Sato, 1969; Malawista, Sato, Creasey & Bensch, 1969) after treatment with vinblastine. Both reports supposed that the crystals were composed from the breakdown product of microtubules, and Bensch and Malawista (1969) actually called them microtubular crystals. The interest in this drug centred around the crystals which it induced, since it was thought that isolation of the crystals might provide a source of pure microtubular material.

With this background, I decided to investigate the effects of vinblastine on the microtubules in the nutritive tubes of Notonecta. Vinblastine destroyed the birefringence in ovarioles, as did treatment with cold and colchicine. Electron micrographs of nutritive tubes, treated with vinblastine, showed that increasing concentrations of the

drug had greater destructive effects on the microtubules, and also, somewhat surprisingly, on the ribosomes. Crystalline structures were induced in the trophic core and nutritive tubes and to a lesser extent in the nutritive cells and follicle cells. These crystals were similar to those reported in mammalian and echinoderm cells (Bensch & Malawista, 1968; Malawista & Sato, 1969).

A number of my observations support the idea of an interconversion of tubules to crystals. First, microtubules disappeared as crystals formed, and secondly, at certain concentrations of vinblastine, and with different times of treatment, microtubules were found adjacent to crystals and in some instances even surrounded by them. The answer is by no means clear, however, since ribosomes are also destroyed by vinblastine and are replaced by crystal lattices. And there are regions of ovarioles treated with vinblastine, where ribosomes are seen aligned in precise rows at the ends of crystals. A similar alignment of ribosomes has been observed by Krishan and Hsu (1969) in the cytoplasm of cells exposed to vinblastine.

These observations indicate that it is quite possible that the ribosomes, or a ribosomal component, may be involved in the formation of the crystals, although it is most unlikely that the crystals are formed from ribosomes alone, since the oocytes of Notonecta rarely contain crystals, after treatment with vinblastine, although they are packed with ribosomes. Nonetheless, there are cases where ribosomes aggregate to form crystal-like structures (Byers, 1966) and also reports of ribosomes forming helical configurations in cells treated with vinblastine and vincristine sulphates (Kingsbury & Voelz, 1969; Krishan & Hsu, 1969).

The composition of the crystals, which are induced by vinblastine, remains unknown. Krishan and Hsu (1971) have demonstrated autoradiographically that the crystals will bind colchicine, a drug known to interact with isolated microtubule protein. From my observations, it seems

likely that the crystals are formed from microtubules, but microtubules are probably not the only component involved in crystal formation.

Although the composition of the crystals is not yet known, there are clues as to their mode of assembly. In this respect, I regard the precise orientation of the crystals in ovarioles as important. In situations where microtubules are arranged in parallel masses, as in the nutritive tubes and the trophic core, and crystals have their long axes similarly aligned. In contrast, where there is no obvious alignment of microtubules, as in the nutritive cells and follicle cells, the crystals are randomly oriented.

Secondly, my observations show that if the microtubules remain intact after pretreatment with low concentrations of colchicine, then treatment with vinblastine induced the formation of crystals composed of regular hexagonal sub-units. If, however, pretreatment with colchicine destroys the microtubules, crystals do not form on the addition of vinblastine, and the tubes contain aggregates of less organised material. Similar aggregates have been observed in the nutritive tubes of Rhodnius, another hemipteran, after treatment with vinblastine alone (Huebner & Anderson, 1970) and Krishan (1970) has described 'ribosome granular material', as well as regular crystals, in mammalian cells exposed to the drug.

The observations are open to two obvious interpretations. The lack of crystals, after pretreatment with colchicine and then vinblastine, could be due to the absence of the microtubules, which may act as a template or skeleton, or even as a seed on which the sub-units assemble, much as microtubule fragments initiate the reconstitution of tubules from a solution of sub-units (Stephens, 1969). Alternatively, it is possible that the binding of high concentrations of colchicine to the sub-units of the microtubules may interfere with the manner of binding of vinblastine which normally

produces crystals. Biochemical evidence suggests that colchicine and vinblastine do not compete for binding sites on microtubule protein (Wilson & Friedkin, 1967; Creasey & Chou, 1968).

Clearly the presence of intact microtubules is essential for the assembly of regular shaped crystals, and I suggest that if the microtubules are not present then the crystal components have nothing on which to assemble, and become evident as the loose feathery material which aggregates in the tubes. Perhaps the aggregation of loosely packed material, as well as regular crystals, in the nutritive tubes of Rhodnius after treatment with vinblastine (Huebner & Anderson, 1970) may be due to the scarcity of microtubules within certain areas of the tubes.

Because of the observations of crystals in tissues after exposure to vinblastine, and the inference that the crystals were, at least in part, microtubular, the question arose - could crystals be produced *in vitro*, and thus allow the collection of microtubular material for biochemical analysis? Supernatants of homogenates of ovaries of Notonecta became cloudy when treated with vinblastine, and a similar precipitation has been reported by a number of groups of researchers (Marantz, Ventilla & Shelanski, 1969; Bensch, Wisniewski & Shelanski, 1969; Olmsted, Karlson, Klebe, Ruddle & Rosenbaum, 1970), all of whom stated that microtubule protein had been precipitated. Marantz and Shelanski (1970) claim to have observed microtubular crystals *in vitro*, by electron microscopy, but my investigation of the white vinblastine precipitates by negative staining and thin sectioning revealed no evidence of crystals; which is precisely as might be expected if intact microtubules are essential for the assembly of crystals.

In my experiments, vinblastine precipitated microtubule protein, which had been isolated by the colchicine binding technique of Borisy and

Taylor (1967). This suggests that the two drugs bind to different sites on the monomer. In addition to precipitating the colchicine binding fraction, vinblastine also caused cloudiness in some of the other protein fractions, indicating that vinblastine does not precipitate specifically microtubule protein, as was originally supposed. Furthermore, electrophoretic analysis of the white precipitate, formed on the addition of vinblastine to homogenates of whole ovaries, showed that there were a number of proteins in the resolubilised precipitate and that one of these comigrated with microtubule protein isolated from the flagellae of Chlamydomonas.

Since I performed this part of my study, Wiesenberg and Timasheff (1970) and Wilson, Ruby and Mazia (1970) have discovered that the precipitating effect of vinblastine on the microtubule protein is mimicked by divalent cations, such as calcium, and that microtubule protein is not precipitated specifically. More recently, Raff, Greenhouse, Gross and Gross (1971) have found that the precipitation of microtubule protein by vinblastine is specific if the precipitation is carried out at 0°C rather than at room temperature.

The telotrophic ovary has proved to be an excellent system for the investigation of various aspects of the biology of microtubules. The experiments with drugs and radioisotopes, performed to date, have either involved the injection of the substances into live animals, or the bathing of excised ovaries for limited periods in a Ringer solution. I considered that an *in vitro* culture technique for telotrophic ovaries would facilitate

more detailed examination of the action of antimitotic drugs and the incorporation of labelled compounds under strictly controlled conditions. Such a technique has been described in the methods section of this thesis. The ability to culture successfully the telotrophic ovary expands considerably the usefulness of this organ in the study of the structure, function and assembly of microtubules; and is being used to investigate the recovery of the microtubular system within the ovaries after treatment with anitmitotic agents.

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- Appendix I -

A massive system of microtubules associated with cytoplasmic movement in telotrophic ovarioles

by

H.C. Macgregor and H. Stebbings

Department of Zoology, The University, St. Andrews, Fife, Scotland

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SUMMARY

The telotrophic ovary of Notonecta glauca glauca consists of 7 ovarioles. Each ovariole comprises, from front to rear, a terminal filament, a trophic region, a pre-follicular region, and a series of 10 to 15 follicles of progressively increasing size. The trophic region is largely syncytial and is made up of polyploid trophic nuclei packed around a central trophic core. The cytoplasm of the trophic core is continuous with the cytoplasm of each oocyte through a system of trophic tubes. There is one trophic tube per oocyte. The trophic nuclei have large nucleoli. There are a few small nucleoli in the oocyte nuclei. The cytoplasm of the trophic core, the trophic tubes, and the oocytes is rich in RNA. Autoradiographs of sections of ovarioles fixed 2 h after injection of ^{3}H-uridine into animals show label over the trophic nuclei only. 8 h autoradiographs show heavy labelling of the trophic region, label over the front ends of the trophic tubes, but little label over the posterior regions of the tubes or the oocyte cytoplasm. Later autoradiographs indicate that label gradually spreads backwards from the trophic core, along the trophic tubes and progressively builds up in the oocyte cytoplasm. These observations are thought to indicate synthesis of RNA in the trophic region and movement of RNA from the trophic core along the trophic tubes to the oocytes. The trophic core and tubes show brilliant positive form birefringence with respect to their lengths. This birefringence can be reduced by keeping animals at 2°C for 12 hours, and eliminated by placing ovarioles in 1% colchicine for 6 hours. Electronmicrographs of thin sections of ovarioles show that trophic core and tubes are densely and uniformly packed with ribosomes

and microtubules. The latter are lined up along the trophic tubes. There are about 30,000 microtubules evident in a TS through a trophic tube 15μm wide. Lengths of microtubules up to 2μm have been observed. Ribosomes are packed between the microtubules but are excluded from regions where the spacing between adjacent microtubules is less than 25nm. The contribution of the trophic region to the oocytes and the role of the microtubules in maintaining or facilitating the movement of ribosomes along the trophic tubes is discussed.

INTRODUCTION

The distinctive feature of a meroistic insect ovariole is that it contains follicular epithelial cells, growing oocytes, and nutritive cells or trophocytes. In a telotrophic ovary, as found for example in the "backswimmer" Notonecta, each ovariole comprises, from front to rear, a terminal filament, a trophic region consisting almost entirely of nutritive tissue, a prefollicular region containing young oocytes, and a germinal region consisting of a linear series of follicles of progressively increasing size. Most of the trophic region is in the form of a syncytium with many large nuclei arranged around a central cylinder of cytoplasm, the trophic core. From the posterior end of the trophic core there extend backwards a number of cytoplasmic processes, the so-called "nutritive cords" (Bonhag 1955). Each cord leads to one oocyte and is the pathway along which nutritive materials are presumed to flow on their way to the oocyte. There is direct cytoplasmic continuity between oocyte and trophic core.

In this paper are certain observations on the flow of material from trophocyte to oocyte in telotrophic ovarioles, the mechanisms which may be responsible for maintaining this flow, and the nature of the material which passes from cell to cell.

A variety of substances have been said to pass from trophocyte to oocyte. Bhandari and Nath (1929) stated that in the red cotton bug Dysercicus cingulatus "nuclear contents of the nurse cells pass along the nutritive roots (cords) in the form of a liquid . . . . . while . . . . . the Golgi elements of the nurse cells remain intact

and flow down the roots (cords) as such, and subsequently enter the egg". Both Schrader and Leuchtenberger (1952) and Bonhag (1955, 1958) have suggested from observations on various Hemiptera that DNA derived from the breakdown of trophocyte nuclei transforms into a Feulgen-negative substance that passes down the nutritive cords into the oocyte cytoplasm; and Bonhag (1955) has described a continuous flow-like pattern of RNA extending from the cytoplasm around the trophocyte nuclei into the trophic core and through the nutritive cords into the oocytes.

In our study of movement of material from trophocyte to oocyte we have examined by autoradiography the synthesis and behaviour of RNA in telotrophic ovarioles. We chose RNA since it is easy to identify and easy to label. In our search for mechanisms associated with such movement we took note of the term "nutritive fiber" (Bhandari and Nath, 1929) which was used to describe thick hyaline fibers spreading out into the cytoplasm of the egg from the entrance point of the nutritive cord, and we noted the many cases where trophocyte/oocyte cytoplasmic bridges have been depicted as filled with a mass of fibrous material (Bhandari and Nath 1929; Bonhag 1955). It is now well recognised that wherever there is movement of cells or within cells there are usually microtubules (Porter 1966), and where these microtubules are present in large numbers, as for example in a mitotic spindle, then they may show in light microscope preparations as fibrous and birefringent masses (Inoué 1964; Bajer 1968). Accordingly we predicted that the fibrous representations of trophocyte/oocyte connections denoted complex and highly concentrated systems of microtubules, and we therefore examined these connections in polarized light and by electron microscopy.

MATERIALS AND METHODS

The animals used in this study were of the species Notonecta glauca glauca (Linn) and were collected from ponds in Tentsmuir Forest, Fife, Scotland.

For examination by light microscopy freshly excised ovaries were fixed for 2 h in 3:1 ethanol acetic acid (Clarke 1851), dehydrated, embedded in wax, and sectioned at 5μ. The sections were stained with gallocyanine (per Swift 1955) washed, dehydrated and mounted in balsam. Other sections were treated prior to staining with a solution of ribonuclease (0.1 mg per ml ribonuclease A in 0.01 M phosphate buffer pH 6.0) for 2 h at 37°C.

For observations in polarized light whole ovarioles were mounted in insect ringer and examined with a Carl Zeiss photomicroscope POL fitted with planapochromatic objectives, a polarizer and an alalyzer. Microtubules are disorganized and their birefringence destroyed by exposure to low temperature (Inoué 1952a; Tilney and Porter 1967) or to colchicine (Inoué 1952b; Tilney 1968). We have therefore compared the birefringence in freshly excised ovarioles with that in ovarioles previously subjected to low temperature and with that in ovarioles previously treated with colchicine. For low temperature treatment live animals were kept in a refrigerator at 2°C for 12 hours. Their ovaries were removed and teased into ovarioles under cold conditions. Single ovarioles were then mounted on slides in insect ringer close alongside ovarioles from untreated animals, and the birefringence in both types of ovariole was compared. For colchicine treatment both ovaries were removed from an animal. One was placed in a solution of 0.5% colchicine in insect ringer. The other was kept in insect ringer without colchicine.

Each ovary was gently teased into its individual ovarioles. At 15 minute intervals after excision of the ovaries pairs of ovarioles, one from the colchicine solution and one from the plain ringer, were mounted on slides and their birefringence examined.

Our autoradiographic studies were carried out as follows. Each of a number of animals was injected with 5µl (5µC) of a solution of uridine generally labelled with tritium (1.2 C/mM), and supplied by the Radiochemical Centre, Amersham, England. The injection needle was inserted through the arthrodial membrane between the 2 most posterior sternites. Animals were killed and their ovaries fixed 2 h, 8 h, 12 h, 24 h, and 3 weeks after injection. The ovaries were fixed for 12 h in Sanfelice's fixative (Darlington and La Cour 1942). They were subsequently washed in running water for 24 h, dehydrated in ethanol and embedded in methacrylate. One-micron sections of ovarioles were cut with glass knives on a Porter-Blum ultramicrotome, and mounted on slides. Methacrylate was removed from the sections with amyl acetate. Sections were then rehydrated, treated with 5% trichloroacetic acid at 5°C for 5 m, dehydrated and air dried from acetone. Mounted sections were coated with Kodak NTB 2 liquid emulsion diluted 1:1 with water. They were then placed in light-tight boxes and left to expose for periods of 20 to 40 days at 18°C. Preparations were developed in Kodak D 19 b developer for 3 min, washed in water, fixed in Kodak Acid Fixer for 5 min, washed again in water, and air dried. Sections were then stained with 0.5% methylene blue on 1% sodium tetraborate (Borax) for 1 to 2 mins. All preparations were dehydrated in ethanol, mounted in balsam, and examined with a Carl Zeiss photomicroscope fitted with planapochromatic objectives.

For electron microscopy single ovarioles were freshly excised into a 5% solution of glutaraldehyde in 0.06 M phosphate buffer at pH 7.3 and 18°C. After 15 minutes in glutaraldehyde the ovarioles were washed briefly in phosphate buffer and transferred to 1% osmium tetroxide buffered to pH 7.3 with veronal acetate (Palade 1952). The ovarioles were then dehydrated in acetone and embedded in Vestopal W. Silver to grey sections were cut with glass knives on a Cambridge Ultramicrotome (A.F. Huxley pattern), mounted on Athene 483 grids without supporting films and doubly stained with uranyl acetate for 5 minutes and lead citrate for 2 minutes (Reynolds 1963). All staining solutions were filtered through a Millipore filter (0.45μ pore size) immediately before use. Sections were examined with a Siemens Elmiskop I (80 kv) at negative magnifications of between 5,000 and 40,000.

OBSERVATIONS

In our descriptions of the ovarioles of Notonecta we have substituted the word "tube" for the term "nutritive cord" since we do not think that the word "cord" suits the situation which we have found.

In an ovariole of Notonecta the trophic core branches at its posterior end into about 20 tubes. These tubes pass backwards and spread outwards in the prefollicular region to become arranged around the periphery of the ovarioles, thus encasing the more anterior follicles like the bones of a corset (Fig. 1, 2, 4). As a rule, each oocyte is served by one tube, and accordingly the number of tubes decreases from front to rear along the ovariole. Tubes extend back-

wards from the trophic core to all follicles which contain eggs without capsules. The connections between trophic tubes and oocytes break down before a capsule forms. There are at least as many tubes evident in transverse sections through the region of the first follicle as there are follicles in the ovariole (Fig. 9). The tubes appear oval in cross section. The width of the tubes in an ovariole varies from 10μm to 12μm. The width of a single tube is roughly constant along its length. The tubes are usually bounded by follicle cells but in some places an oocyte may form the inner boundary of a tube. The trophic core and all tubes have a streaky or fibrous appearance when seen in longitudinal section (Fig. 2). In transverse section however, they appear uniformly granular.

Five micron sections stained with gallocyanine and without prior digestion in ribonuclease served to show the distribution of RNA in ovarioles. All cytoplasm in the trophic region, including the trophic core, all tubes, and the cytoplasm of all oocytes stained intensely (Figs. 1, 2). Most of the nuclei in the trophic region and all follicle cell nuclei had large nucleoli which stained strongly. In all our stained preparations the nuclei of oocytes occupying the first 2 or 3 follicles appeared empty apart from thin strands of chromosomal material. Nuclei of the more posterior oocytes contained 3 to 5 small nucleoli. In preparations pre-treated with ribonuclease only nuclear chromatin was stained.

When a single ovariole is mounted in insect ringer and examined between crossed polaroids in a polarizing microscope all tubes show strong positive form birefringence with respect to their length (Fig. 4). The middle of the trophic core is likewise strongly

birefringent, but towards the edges of the core the birefringent material becomes more diffuse and faint (Fig. 3). There are a series of narrow lines of birefringence radiating laterally into the trophic tissue from the trophic core. Most tubes appear evenly birefringent throughout their lengths, although a gentle twisting of the birefringent material in the tubes is sometimes evident. In a few instances we have seen ovarioles burst under pressure between slide and coverglass. When this happens some of the tubes may become partially isolated from the rest of the tissue. These isolated tubes do not break, nor do they disintegrate; they remain compact and brightly birefringent.

The trophic core and tubes in ovarioles subjected to cold treatment show weaker birefringence than those in untreated ovarioles (Fig. 5).

Ovarioles treated with 0.5% colchicine for 6 h show no birefringence so that neither trophic core nor tubes are discernible in polarised light (Fig. 6). Other effects of colchicine are visible however. The more obvious of these are swelling of the neck of the trophic region, and disorganization of the trophic region so that trophic nuclei invade the trophic core. The orderly arrangement of trophocytes, oocytes, and tubes in the pre-follicular region disappears, the cells intermingle, and everything tends to flow backwards to fill the spaces originally occupied by the trophic tubes.

In our autoradiographs of sections of ovarioles fixed 2 h after injection of ^{3}H-uridine only the nucleoli of nuclei in the trophic region were labelled. In autoradiographs of sections from 8 h fixations nuclei and cytoplasm in the trophic region, including the trophic core

were labelled. Tubes were lightly labelled towards their anterior ends, but the labelling of the tubes alongside the more posterior follicles was scarcely above background. Both nuclei and cytoplasm of oocytes in the follicular region were unlabelled. In autoradiographs made 12 and 24 hours after injections the trophic region was heavily labelled, all tubes were labelled, and the cytoplasm of all oocytes was labelled above background (Figs. 7, 8, 9). In autoradiographs of transverse sections through ovarioles which had been exposed to ^{3}H-uridine for 12 h or more a few of the trophic tubes were quite unlabelled (Fig. 9).

We employed the technique of autoradiography because we wished to see if RNA synthesized in the trophic region moved backwards along the tubes and accumulated in the oocytes' cytoplasm. We predicted that if this were the case then soon after injection of isotope into the insect the trophic region should be labelled, the oocytes unlabelled, and such labelling as was present over the tubes should be heaviest at the front near the trophic region and progressively lighter towards the rear. A little later the tubes should be more evenly labelled along their lengths. Later still, when the supply of ^{3}H-uridine was exhausted and no more "hot" RNA was being synthesized in the trophic region, the labelling over the tubes should be heaviest towards the rear and lightest at the front. Finally, everything should be unlabelled except the oocytes' cytoplasm.

To test these predictions grain counts were made over 2 areas of the trophic core, over portions of tubes alongside the first 6 follicles, and over the cytoplasm of the 7th oocyte in ovarioles fixed

at 2, 8, 12, and 24 h, and 3 weeks after injection of isotope into the animals. Each count was made over a square covering 150μ^2. Our complete results are shown in table 1 and a sample of our results is presented in histogram form in Fig. 10. We conclude from our observations that the oocyte nucleus itself synthesises little, if any, RNA, that the follicle cells synthesise RNA some of which may pass directly to adjacent oocytes and some to adjacent tubes, and that most of the RNA synthesized in the trophic region is moved along the tubes to the oocytes' cytoplasm. We think that the tube contents move at a rate of at least 20μm per hour.

In our electron microscope studies we have concentrated upon structure in the trophic region and in the tubes. Peripheral trophic tissue consists of poorly defined cells with large nuclei (Fig. 11). Each nucleus is highly irregular in shape and has one or two large nucleoli. The most striking feature of this tissue is the chaotic nature of the cell boundaries and membranes. Cell boundaries are usually impossible to trace, the distribution of nuclei in the cytoplasmic compartments in haphazard, and much of the tissue is syncytial. Cytoplasm in this region is rich in mitochondria and ribosomes. There is little endoplasmic reticulum and Golgi material is rare. Cellular areas are interspersed with tracts of cytoplasm which contain ribosomes, occasional mitochondria, and parallel microtubules (Fig. 11). These tracts are less densely packed with ribosomes than cytoplasm around the trophic nuclei. The tracts are relatively sparse near the periphery of the trophic tissue, but numerous near the trophic core. As one looks inwards towards the trophic core the close-packed complexity of the peripheral tissues gives way to a random scatter of large degenerate

nuclei in a vast sea of cytoplasm (Fig. 12). This cytoplasm is strikingly uniform and consists almost exclusively of a dense mixture of ribosomes and microtubules. So numerous are the microtubules that in some sections through regions near the edge of the trophic core we have seen microtubules form flow patterns around and between trophic nuclei.

The fine structure of the trophic tubes is truly remarkable. Each tube is filled with a vast array of microtubules and ribosomes. The microtubules lie roughly parallel to one another and all of them run lengthwise along the tube (Figs. 13, 15, 16). They are about 200 Å in width. The distance between adjacent microtubules varies from apparent zero, where two tubules touch, to about 50nm. We estimate that in a transverse section of a trophic tube 15μm wide there are about 30,000 microtubules (Fig. 14). The desnity of packing of microtubules seems roughly constant throughout the length of a trophic tube. We have seen lengths of individual tubules up to 2μm long. The general courses of the microtubules are in straight lines but each tubule is finely wavy in appearance (Figs. 15,16). Where a part of a follicle cell or other large object projects from the wall of a trophic tube and partially obstructs the tube, the microtubules bend around the obstruction and are relatively scarce on either side of it.

The number and packing to ribosomes in trophic tubes varies from animal to animal and show slight local variations in individual tubes (Figs. 15,16). Where ribosomes and microtubules are intermixed the ribosomes are arranged in streams running between lines of microtubules. Ribosomes are most densely packed where there are no microtubules (Fig. 15). We cannot at the moment define the precise structural or spatial relationship between the ribosomes and the microtubules, but our electron micrographs indicate that contact between ribosome and microtubule is rare. Indeed, as a rule the ribosomes are largely

excluded from regions where the lateral spacing between the microtubules is less than 25nm, and in transverse sections microtubules often appear ringed by ribosomes at a distance of about 30 nm (Fig. 14).

DISCUSSION

The evidence for movement of materials, including RNA, from trophocytes to oocytes in telotrophic ovaries is almost incontrovertible. The points which we would add to the classical description of the telotrophic ovariole, however, are as follows. First, the oocyte nucleus itself synthesizes little if any RNA; yet newly synthesized RNA appeared in the oocyte cytoplasm in the course of our experiments. This newly synthesized RNA was probably imported, and the most likely import channel is the trophic tube which connects the oocyte to the trophic core. Secondly, our observations on the pattern of labelling of the trophic tubes after different periods of exposure to ^{3}H-uridine are most easily explained by a continual stream of newly synthesized RNA from trophic tissue to oocyte via the trophicore and the trophic tubes. Thirdly, most oocytes store ribosomes during oogenesis. Those oocytes that make their own ribosomal RNA for storage have their ribosomal genes amplified and are multinucleolate during the greater part of oogenesis (Brown, 1968). The oocytes of Notonecta are not multinucleolate, and at no stage in their development do they show evidence of specific gene amplification. We may suppose that these oocytes, lacking the mechanism for high speed production of large amounts of ribosomal RNA, rely on ribosomes imported fron the trophic tissue via the trophic tubes. That the trophic tissue is primarily engaged upon production of ribosomes for export is indicated by the large nucleoli

of the trophic nuclei, the abundance of ribosomes in the cytoplasm, and the lack of endoplasmic reticulum and Golgi, components usually associated with the immediate involvement of RNA in protein synthesis.

What flows down the trophic tubes? The most obvious structures are ribonucleoprotein particles which, on account of their size and appearance in electron micrographs, we have called ribosomes. The only large objects which we have seen in the trophic tubes are mitochondria and these are sparse. We do not know if they move or not. We consider it significant however that we have seen no other class of large objects in trophic tubes, no nuclei, no large aggregates of nuclear material, no fragments of membrane or vesicles, and this in spite of the fact that the trophic core contains all manner of debris from the breakdown of trophic tissue.

Let us now consider the microtubules and the part which they may play in maintaining the flow of RNA through the trophic tubes. The microtubules which we have described in the trophic core and tubes of a telotrophic ovariole are tubules in the sense that they appear in transverse section as a dark ring surrounding a less dark central area. They are about 20nm in width. They are lined up along the length of each trophic tube. They are present in sufficient numbers to produce strong form birefringence when the tubes are examined in polarized light and this birefringence is absent from ovarioles pretreated with cold or colchicine. In all these respects they are essentially comparable to the microtubules discussed several years ago by Porter (1966) and since recognised in a wide variety of situations. Moreover, like microtubules in other places the microtubules of trophic tubes clearly have something to do with the movement of cytoplasm and possibly have something to do with maintenance of form.

In his review on cytoplasmic microtubules and their functions Porter (1966) interprets a wide range of observations on microtubules as suggesting that they may be effective in the development and maintenance of asymmetrical cell forms, in limiting channels through which cytoplasm moves, or in providing the motive force for cytoplasmic streaming. A question which emerges from this review is essentially do microtubules cause cytoplasmic movement or facilitate it? We have no fresh evidence to offer towards the solution of this problem but we think that the following points are worth stressing.

First, the microtubules of the trophic core or tubes show no regular arrangement with respect to one another. In appearance and distribution they compare with the microtubules in the endoplasmic strands of coenocytic marine algae (Sabnis and Jacobs 1967) and in nerve axons (Porter 1966). In each of these cases the microtubules occupy a zone through which cytoplasm of cytoplasmic components are seen or presumed to flow. In none of these cases is there an obvious need for skeletal support from microtubules. In other situations however, such as axopodia of heliozoans (Tilney and Porter 1965) the cytopharyngeal basket of ciliates (Tucker 1968), or the heads of elongate sperm or spermatids (Robinson 1966; McIntosh and Porter 1967; Kessel 1967) the microtubules have a definite, sometimes helical, arrangement, and whatever part they may play in moving cytoplasm they clearly perform some mechanical supporting role.

Secondly, the arrangement of nuclei in the trophic region is orderly. They are closely packed around the trophic core but they do not encroach upon it; yet there is nothing other than microtubules to prevent such encroachment. In the trophic region however, the microtubules are often orientated around and between nuclei and other obstacles and usually lie along the expected direction of cytoplasmic

flow. This would seem to indicate that microtubules, whilst functioning as a skeleton to prevent clogging of the channels through which material flows, are also intimately concerned with directing and/or maintaining the flow.

That the microtubules which we have described do preserve the trophic core and prevent large objects from entering and blocking the trophic tubes is suggested by the fact that the most immediate and dramatic consequences of placing an ovariole in colchicine is obliteration of the trophic core and disappearance of the trophic tubes. Essentially, having destroyed the microtubules nothing remains to prevent surrounding nuclei and cells from invading trophic core and tube.

In our opinion the microtubules in ovarioles of Notonecta have one definite function. They facilitate a flow of ribosomes from trophocytes to oocytes via trophic core and trophic tubes by keeping these regions open. We think however, that they also direct the flow and we are searching for evidence of their involvement in the maintenance of flow.

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Table 1.

Number of silver grains over an area of 150 μm^2 in two regions of of the trophic core, regions of nutritive tubes alongside successive follicles and cytoplasm of the 7th follicle, in preparations fixed at different times after H^3-uridine injection. (Each number is the mean of four counts, each count being made over a different area). The number "0" indicates that the grain count was not significantly above background.

Fixation time	Trophic core		Label above nutritive tubes opposite follicles						Cytoplasm of 7th follicle
	Front	Rear	1	2	3	4	5	6	
2 h			UNLABELLED						
8 h	12	2	1	2	1	1	1	1	0
	18	8	2	3	0	1	0	0	0
	10	7	1	0	0	0	0	0	0
	11	8	2	1	2	2	0	0	0
12 h	20	13	8	9		5			4
	19	14	11	8	3	3	7	9	3
	18	14	11	9	7	6	6		5
	20	13	13	10	6	12	9	7	4
24 h	83	56	22	14	17	20	20	16	9
	44	39	11	13	12	10	9	7	5
	41	20	12	7		11	10	13	7
	38	24	13	11	11	12	9	7	4
3 weeks	6	7	6	6	7	9	12	13	18
	5	7	7	7	7	-	12	13	21

Legends to figures

Fig. 1. Longitudinal section (LS) through the trophic region of an ovariole stained with gallocyanine. The anterior tip of the ovariole is to the left. Trophic core, c; trophic tubes, t.

Fig. 2. LS through part of a trophic tube, stained with gallocyanine. Note the fibrous appearance of the tube contents.

Fig. 3. Photomicrograph in polarized light of the whole trophic region of an ovariole. The bright object to the left is the terminal filament. The trophic core is strongly birefringent and breaks up at its posterior (right hand) end into a number of trophic tubes.

Fig. 4. Photomicrograph in polarized light of part of the trophic region, the prefollicular region, and the first follicles. About 14 strongly birefringent trophic tubes emerge from the trophic core (top), bow outwards around the prefollicular region (middle) and run back alongside the follicles.

Fig. 5. Photomicrograph in polarized light of the front regions of 2 ovarioles. The one on the left came from an animal which had been kept at 2°C for 24 hours. The one on the right came from an animal which had been kept at 18°C.

Fig. 6. Photomicrograph in polarized light of the frond regions of 2 ovarioles. The one on the right had been kept in a 1% solution of colchicine in insect Ringer for 6 hours. The one of the left was kept in Ringer without colchicine.

Figures 7, 8 and 9 are autoradiographs of parts of ovarioles fixed 24 hours after injection of ^{3}H-uridine into the animals.

Fig. 7. LS through part of the trophic region and the prefollicular region. Trophocyte nucleoli, n; trophic core, c; and trophic tubes (t) are heavily labelled. The cytoplasm of small oocytes (o) is lightly labelled, but the oocyte nuclei are unlabelled.

Fig. 8. LS through the 5th and 6th follicles in an ovariole. Trophic tubes (t) are heavily labelled. Oocyte cytoplasm is lightly labelled, but the labelling over oocyte nuclei is scarcely above background.

Fig. 9. TS through the 3rd follicle. Sections of 15 tubes are visible. All but 3 of the tubes (arrowed) are labelled.

Fig. 10. These histograms represent grain counts from autoradiographs of different regions of ovarioles fixed at various times after injection of ^{3}H-uridine into animals. Each histogram is from one LS of a single ovariole and is constructed from data in the line marked with an asterisk in table 1.

Fig. 11. Electron micrograph of a section through part of the trophic region. Trophocyte nucleolus, n; tracts running around and between trophic nuclei and containing ribosomes and microtubules, tr.

Fig. 12. Electron micrograph of a section through part of the trophic core. This region contains ribosomes, microtubules, scattered mitochondria, and large degenerate trophic nuclei (n).

Fig. 13. Electron micrograph of an LS through part of a trophic tube. The tube contains only ribosomes and numerous longitudinally arranged microtubules. The tube is bounded by the membrane of the follicle cells.

Fig. 14. Electronmicrograph of a TS through part of a trophic tube. Note the clear zone which surrounds many of the microtubules (arrows) and the apparent variation in the diameters of the microtubules in this section.

Fig. 15. LS through part of a trophic tube showing a bank of microtubules (upper half) with relatively few ribosomes between them, and a region packed with ribosomes but clear of microtubules.

Fig. 16. Oblique section through part of a trophic tube from another animal showing large numbers of closely packed microtubules with a few ribosomes scattered amongst them.

- APPENDIX II -

Influence of vinblastine sulphate on the deployment of microtubules and ribosomes in telotrophic ovarioles

by

H. Stebbings

Department of Zoology, The University, St. Andrews, Fife, Scotland.

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SUMMARY

Ovarioles of Notonecta glauca glauca have been treated with vinblastine sulphate (VBL), primarily to examine the effects of the drug on the microtubules which pack the trophic core and nutritive tubes. 10^{-4} M VBL had little effect on the microtubules. VBL at 10^{-3} M reduced the number of microtubules in nutritive tubes, while after 2×10^{-3} M VBL microtubules were rare. All 3 concentrations of VBL induced the formation of crystalline structures in the trophic core and nutritive tubes, and to a lesser extent in the nutritive cells and follicle cells. In transverse sections crystals appeared to be composed of distinct hexagonal units and in longitudinal sections crystals showed as dark parallel lines with darker dots at regular intervals along their length. With 10^{-4} M VBL, the crystals were compact and close to the walls of nutritive tubes, but after treatment with stronger concentrations of VBL the crystals were web-like and scattered over cross sections of tubes.

In addition to VBL having a destructive effect on the microtubules in the trophic core and nutritive tubes, there was also a reduction in the number of ribosomes. This overall reduction in ribosomes quantity could not be attributed to the inhibition of RNA synthesis by VBL.

In regions of ovarioles where microtubules were arranged in parallel masses, as in the nutritive tubes and trophic core, the long axes of crystals were always similarly aligned. Otherwise crystals were randomly orientated. The possible mode of formation, and composition, of crystals is discussed.

INTRODUCTION

Notonecta glauca glauca has a telotrophic meroistic ovary. Nutritive cells or trophocytes surround an anterior trophic core of cytoplasm and nutritive tubes pass back from this to each oocyte in the ovariole. Recently Macgregor & Stebbings (1970) have shown that the nutritive tubes are packed with microtubules and ribosomes, and they have demonstrated a movement of RNA from the trophic region to the oocytes via the nutritive tubes. The packed microtubules render both the trophic core and the nutritive tubes strongly birefringent and this birefringence is destroyed by cold and colchicine.

Vinca alkaloids, such as vinblastine and vincristine sulphates, imitate the mitotic arrest properties of cold and colchicine. Bensch & Malawista (1969) have found that vinblastine (VBL) destroys the majority of microtubules in both L.-strain fibroclasts and human leucocytes and that crystals subsequently appear in the cytoplasm of treated cells. Similarly, VBL induces the formation of birefringent crystals in unfertilized oocytes of the starfish Pisaster ochraceous (Malawista & Sato, 1969). In both reports the authors suggest that the crystals are formed from microtubules and that isolation of the crystals should provide a means of obtaining relatively pure microtubule protein.

Wisniewski, Shelanski & Terry (1968) found that VBL caused an initial loss of tubules from the axoplasm of neurons followed by a proliferation of 10 nm neurofilaments. They propose an interconversion of tubules to filaments as a consequence of the presence of the alkaloid.

In addition to alterations in cell structure there are also reports that vinblastine, vincristine and colchicine interfere with the synthesis of nucleic acids and hence ultimately protein synthesis in cells (Creasey & Markiw, 1965; Desjardins, Grogan, Arendell & Busch, 1967).

I chose to examine the effects of VBL on the contents of the trophic tubes in ovaries of water bugs - aquatic Hemiptera - because the system of microtubules therein is so massive, because only 2 cell components are present; microtubules and ribosomes, and because I wanted to explore the effects, on the trophic tubes, of substances which altered microtubule structure and arrangement.

MATERIALS AND METHODS

Notonecta glauca glauca (Linn.) were caught in a pond in Tentsmuir Forest, Fife. Ovaries were bathed in solutions of vinblastine sulphate ('Velbe', Lilly) in insect Ringer, prior to electron microscopy and examination in polarized light.

For observations in polarized light, ovarioles were mounted in Ringer and examined with a Carl Zeiss photomicroscope POL fitted with planapochromatic objectives, a polarizer and an analyser. Microtubules aligned in parallel masses always exhibit birefringence when examined in polarized light. Destruction of the microtubules removes this birefringence (Inoué 1952a, 1952b; Tilney & Porter, 1967; Tilney, 1968). It was therefore possible using this technique to monitor the overall effect of vinblastine on the microtubules in the trophic core and nutritive tubes of Notonecta. Excised ovaries

were bathed in 10^{-4} M, 10^{-3} M and 2×10^{-3} M VBL, and also in insect Ringer. Vinblastine treated ovarioles were examined at intervals and compared with control ovarioles.

For electron microscopy whole ovaries were bathed for 6 hours in 10^{-4} M, 10^{-3} M and 2×10^{-3} VBL, in insect Ringer. Single ovarioles were then removed and fixed in a 5% solution of glutaraldehyde in 0.06 M phosphate buffer at pH 7.3 for 15 min. and transferred to 1% osmium tetroxide buffered to pH 7.3 with veronal acetate (Palade, 1952). The ovarioles were dehydrated in acetone and embedded in Vestopal W. Silver to grey longitudinal and transverse sections were cut with glass knives on a Cambridge Ultramicrotome (A.F. Huxley pattern), mounted on Athene 483 grids and doubly stained with uranyl acetate for 5 min. and lead citrate for 2 min. (Reynolds, 1963). The staining solutions were all filtered through Millipore filters (0.45µm pore size) immediately before use. Sections were examined with a Siemens Elmiskop I electron microscope.

Experiments to examine the effect of VBL on the synthesis of RNA in ovarioles were performed as follows. One of the 2 ovaries from an animal was placed in 1 ml 10^{-3} M VBL, and the other in 1 ml of Ringer solution containing 7×10^{-3} M sucrose. Ovaries were bathed in VBL and Ringer for periods of 0.5, 1.0 and 1.75 h and then 10µl of a solution of uridine generally labelled with tritium (Radiochemical Centre, Amersham, England), were added to each and incubated at 20°C for 3 h. The ovaries were fixed in ice-cold trichloroacetic acid for 10 min. and washed repeatedly in cold 70% ethanol. Single ovarioles were dried in glass scintillation vials and digested with NCS reagent (Nuclear Chicago) for 12 h at 40°C. Scintillation fluid was added and the vials counted with and without an external

barium standard, using a liquid scintillation counter (Nuclear Chicago). The number of disintegrations per min (dpm) per ovariole from VBL-treated ovarioles was compared with that from control ovarioles.

OBSERVATIONS

The brilliant form birefringence of the trophic core and nutritive tubes has already been described (Macgregor & Stebbings, 1970). 10^{-4} M VBL had no effect on this birefringence. Treatment for 6 h with 10^{-3} M VBL eliminated birefringence from the trophic core and the tubes showed weaker birefringence than those in control ovarioles (Fig. 1). After 2×10^{-3} M VBL for 6 h, birefringence was lost altogether.

In ovarioles treated with 10^{-3} M VBL for 6 h the nutritive tubes were packed with ribosomes, as in untreated ovarioles, but in many cases the parallel system of microtubules had disappeared leaving arcs and circles of ribosomes which previously surrounded individual microtubules.

Crystalline structures were conspicuous in the nutritive tubes, (Fig. 2, 4) especially close to the tube wall, and sometimes touching it. These crystals were rare in the oocytes, but did occur in the cytoplasm of surrounding follicle cells (Fig. 2). In the nutritive tubes the long axes of the crystals were usally parallel to the tube walls. The sizes of crystals ranged up to 1μm across and 4μm in length. In cross section the crystals had a honeycomb appearance (Fig. 3). Each hexagonal unit had an internal diameter of approximately 18 nm. The rim was approximately 6 nm wide and in many cases tubular sub-units could be seen in the rim. Crystals in longitudinal section appeared

as dark parallel lines 25 - 30 nm apart with darker dots at intervals of 20 nm along their length (Fig. 4). Dots on adjacent rows were not positioned in register but slightly out of step.

Although in most cases VBL had a destructive effect on microtubules, in some nutritive tubes intact microtubules co-existed with crystals (Fig. 4). Further, in similarly treated tubes, individual microtubules were surrounded not by ribosomes, as in the controls, but by loose crystal lattice (Fig. 5).

Treatment with 10^{-3} VBL destroyed microtubules in the trophic region of ovarioles. Crystals, of similar size to those in nutritive tubes, were scattered amongst ribosomes in the trophic core, with their long axes parallel to the long axis of the ovariole. Randomly oriented crystals were found in the cytoplasm of nutritive cells. The quantity of ribosomes in the nutritive cells was greatly reduced by VBL, while some of the remaining ribosomes were aligned in rows. These rows of ribosomes were sometimes continuous with the crystals themselves (Fig. 6). There was an increase in the amount of rough endoplasmic reticulum in nutritive and follicle cells after VBL treatment (Fig. 2).

Microtubules were rarely seen in nutritive tubes after 2×10^{-3} M VBL treatment for 6 h (Fig. 7, 8). The ribosome number was also reduced and the tubes appeared empty in comparison with the controls (Fig. 7, 8). Crystals were larger with less well defined boundaries. Open lattice networks with crosslinks of varying lengths, cover much of the nutritive tubes' cross sections (Fig. 8). Unlike the compact crystals these networks were not composed of regular hexagonal units. The long axes of the crystals in the nutritive tubes

were parallel to the tube walls while smaller crystals in the cytoplasm of follicle cells were randomly orientated.

Treatment with 10^{-4} M VBL for 6 h had a relatively slight effect on the number and distribution of microtubules and ribosomes in nutritive tubes. Crystals were evident around the inside of tube walls. The crystals were usually separated from the tube wall by ribosomes but were sometimes in contact with it. Microtubules could be seen close to crystals. The sub-units of the crystals were the same size as those produced by other VBL concentrations. Dark membrane structures up to 0.25μm diameter were seen in the nutritive tubes.

Levels of 10^{-3} M VBL produced an 87% inhibition of incorporation of uridine into RNA. The inhibition of RNA synthesis decreased with longer exposure to VBL. This suggests that the synthetic process which is initially inhibited by the drug, may partially recover. Disintegrations per minute per ovariole from VBL treated and control ovaries are shown in Table 1.

Table 1.

Effects of different durations of VBL pre-treatment on the incorporation of ^{3}H-uridine into ovarioles. (Each number is the mean count of 7 ovarioles)

Duration of VBL prior to the addition of ^{3}H-uridine	Control ovariole in Ringer dpm	Ovariole in 10^{-3} M VBL dpm	Treated as % of control
0.5 hr	27,491	3,590	13.1
1 hr	39,203	6,159	15.7
1.75 hr	28,695	13,538	47.2

DISCUSSION

Vinblastine sulphate destroys the microtubules in nutritive tubes of Notonecta as did cold and colchicine (Macgregor and Stebbings, 1970). The observations show that increased concentrations of vinblastine have a greater destructive effect on the microtubules in nutritive tubes and that the number of ribosomes in the tubes is also reduced by this drug. The spacing and number of microtubules after 10^{-4} M VBL treatment is similar to that in untreated material. In tubes treated with 10^{-3} M VBL the number of microtubules is reduced. After exposure to 2×10^{-3} M VBL microtubules are rare.

The VBL-induced crystals in the nutritive tubes of Notonecta are similar in structure to those produced by VBL in mammalian cells (Bensch & Malawista, 1968; 1969). Increased concentrations of VBL produce larger crystals in the nutritive tubes. After 10^{-4} M VBL treatment the crystals are compact and close to the tube walls, while after 2×10^{-3} M VBL the crystals spread over much of the tubes' cross sections in a web-like fashion. Polarizing-light microscopy and electron microscopy show that VBL destroys microtubules in the trophic region before it affects those in nutritive tubes. I do not think that this reflects a difference between the microtubules of the trophic region and those of the trophic tubes. It is more likely to be a consequence of the relative impermeability of the nutritive tube wall to VBL.

The composition and mode of formation of the crystals is not yet known. Bensch & Malawista (1968; 1969) have suggested that crystals arise directly from microtubules, because crystals form as microtubules disappear and also because of the similarity in size and shape between microtubules and crystal sub-units. The close proximity of crystals and

microtubules in some nutritive tubes, after VBL treatment, supports this idea of a direct conversion.

Microtubules disappear and crystals form; but this does not occur at a distinct concentration of VBL, or treatment period, and it is therefore worth investigating intermediate stages for clues as to crystal composition. After 10^{-3} M VBL treatment for 6 h, most of the microtubules have disappeared from nutritive tubes, leaving circles and arcs of ribosomes which previously surrounded tubules. In some tubes, however, microtubules remain and the ribosomes are replaced by a loose crystal lattice. In the cytoplasm of nutritive cells after a similar treatment, rows of ribosomes can often be seen parallel to, and touching, crystals. Stronger concentrations of VBL produce larger crystals, markedly fewer microtubules and also a considerable reduction in the quantity of ribosomes within the nutritive tubes.

The proliferation of rough endoplasmic reticulum in nutritive and follicle cells after treatment with VBL probably signifies an increase in protein synthesis in these cells. Membranes are rarely found in nutritive tubes, which are essentially a transport system for ribosomes. Krishan & Hsu (1969) have suggested that extra protein may be produced to compensate for that which is probably incorporated into crystals.

The observations reported here show that VBL reduced the level of incorporation of ^{3}H-uridine into ovarioles, just as it has been shown to inhibit incorporation into Ehrlich ascite carcinoma cells (Creasey & Markiw, 1964). This could account for a reduction in the number of ribosomes in nutritive cells and nutritive tubes after VBL treat-

ment. VBL, however, also greatly reduced the number of ribosomes packing the oocytes, and this cannot easily be explained by an inhibition of RNA synthesis, since the oocytes do not make their own ribosomes, but import them from the trophic region by way of the nutritive tubes (Macgregor & Stebbings, 1970). The overall reduction in the number of ribosomes could result only from the destruction of the ribosomes themselves by VBL.

The reduction in ribosomes quantity, their replacement by crystal lattices around microtubules, and their lining up into rows which appear to be continuous with the filaments of the crystals, suggests that the ribosomes, or ribosomal components, may be involved in crystal formation. Clearly the crystals, in ovarioles, are not formed from ribosomes alone, because the oocytes, although rich in ribosomes, have very few crystals. It is nonetheless well known that ribosomes can, and do, aggregate to form crystal-like structures (Byers, 1966), and that ribosomes assume helical arrangements on treatment with vinblastine and vincristine (Kingsbury & Voelz, 1969; Krishan & Hsu, 1969).

I suggest that the orientation of crystals is important. In situations where microtubules are arranged in parallel masses, as in the nutritive tubes and trophic cores, crystals have their long axes similarly aligned. In contrast, where there is no obvious alignment of microtubules, as in the nutritive cells and follicle cells, the crystals are randomly oriented. The question of crystal composition remains. Possibly the crystals are formed entirely from microtubule protein since VBL destroys microtubules and induces the formation of crystals and also because crystals form close to, and around, intact microtubules and are rare in regions lacking microtubules.

Alternatively it is possible that components of both ribosomes and microtubules contribute to the formation of crystals. If this is the case, then the crystals are not such a pure source of microtubule protein as has been anticipated. Certainly there are cases where VBL induces the proliferation of microfilaments and not the formation of crystals (Wisniewski et al., 1968).

Recent experiments have shown that vinblastine precipitates microtubule protein from supernatants obtained from homogenates of tissues rich in microtubules (Marantz, Ventilla & Shelanski, 1969; Olmsted, Carlson, Klebe, Ruddle & Rosenbaum, in press). In some cases, sectioned and negative contrast preparations of precipitates showed features resembling the crystalline structures produced in vivo after treatment with VBL, (Bensch, Marantz, Wisniewski & Shelanski, 1969; Marantz & Shelanski, 1970). These structures have been called microtubule crystals. My own preliminary observations show that vinblastine produces a white precipitate from supernatants of homogenates of Notonecta ovaries, but that this precipiate shows no crystalline or lattice structure when examined by light or electron micrographs. It may be that VBL precipitates several proteins, some of which are not microtubular and that crystals appear only in supernatants of tissues where microtubules contribute a fraction of the total protein large enough to show as a major band upon gel electrophoresis. It would be of interest to know (a) how many proteins are precipitated by VBL, (b) how many of these are involved in the formation of crystals, and (c) whether any of these proteins are derived from ribosomes.

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Legends to figures

Fig. 1. Photomicrograph in polarised light of the anterior region of 2 ovarioles. The ovariole on the left has been bathed for 6 h in 10^{-3} M VBL. The one on the right was kept in Ringer.

Figs. 2, 3, 4, 5 and 6 are electron micrographs of ovarioles after treatment for 6 h with 10^{-3} M VBL.

Fig. 2. Transverse section through part of an ovariole showing an oocyte (Oc), and a nutritive tube (nt) surrounded by follicle cells. Crystals (c) can be seen in the tube and the follicle cells. In the tube the crystals are cut in transverse section, while in the follicle cells crystals are randomly oriented.

Fig. 3. T.S. through a crystal, showing the hexagonal sub-units. In some regions fibrils can be seen in the rim of these sub-units.

Fig. 4. L.S. of a nutritive tube, showing the coexistence of microtubules and crystals. In such sections of nutritive tubes the crystals, and microtubules when present, are always seen in longitudinal section.

Fig. 5. T.S. through part of a nutritive tube showing microtubules surrounded by an extensive crystal lattice. There are few contacts between crystals and microtubules.

Fig. 6. Section through the cytoplasm of a nutritive cell. Rows of ribosomes appear to be in continuity with the crystal, which is seen in L.S. There is an out-of-step arrangement of ribosomes in adjacent rows.

Figs. 7 and 8 are electron micrographs of ovarioles after 6 hrs. Treatment with 5×10^{-3} M VBL.

Fig. 7. T.S. through part of a nutritive tube showing a crystal cut in T.S. In comparison with controls, very few microtubules can be seen, and the quantity of free ribosome is correspondingly reduced.

Fig. 8. Crystal structures spread over much of the T.S. of the nutritive tube. In places the crystal structure is loose and web-like (arrowed) which contrasts with the regular hexagonal sub-units of compact crystals. In this tube there is an almost total absence of free ribosomes.

- Appendix III -

Maintenance of insect ovarian microtubular structure and function *in vitro*.

The morphology and ultrastructure of the telotrophic ovary of *Notonecta glauca glauca* Linn., and the system whereby ribosomes are transported from trophocytes to oocytes has been described by Macgregor and Stebbings[1]. One of us, (H.S.) has been involved in an investigation of the extensive system of microtubules in the nutritive tubes, especially with regard to the effects of antimitotic agents on these organelles[2]. Previous experiments with drugs and radioisotopes have involved the injection of the substances into live animals. We considered that an *in vitro* culture technique, for telotrophic ovaries, would facilitate more detailed examination of the action of antimitotic drugs and the incorporation of labelled compounds under strictly controlled conditions. The present study describes such a technique.

Ovaries were removed under Locke's sterile insect Ringer, washed three times in sterile Ringer, and finally in Grace's Modified Insect Medium (Grand Island Biological Company). The culture chambers used were 5.5 cms diam. sterile plastic petri dishes (Nunclon; Steriling Ltd.), the bases of which were scratched with a scalpel blade. By scratching at right angles, points of plastic were raised, and these acted as rafts which elevated the ovaries from the base of the dish, thus allowing free circulation of medium around the ovaries. The roughened base also served to anchor the ovary during the changing of media. The excised and washed ovaries were transferred to the rafts in the petri dishes, which contained 2.5 mls of insect medium to which had been added Crystamycin (0.16mg/ml : Glaxo) and Achromycin (0.02mg/ml : Lederle).

During long periods of culture, the medium in each dish was changed routinely every twelve hours. All procedures were performed in a portable sterile workbench (Microflow).

The structural integrity of the ovaries during culture periods of up to 2 days was examined in polarised light and by electron microscopy. Structures composed of packed microtubules are invariably birefringent [1,3,4], and we have utilised the intensity of birefringence as a rough indication of the state of the microtubules in the nutritive tubes. The trophic cores and the nutritive tubes were strongly birefringent throughout culture periods of 2 days, indicating microtubule preservation. Electron micrographs of nutritive tubes after 2 days in culture were indistinguishable from those of freshly excised material [1].

The synthesis of RNA and its transport down the trophic tubes has been demonstrated *in vivo* [1]. It has been reported, however, that the salivary glands of *Drosophila* failed to synthesise ribosomal RNA *in vitro* [5]. We therefore thought it important to determine for our culture system a) whether RNA was continually synthesised, b) if rRNA was made, and c) whether it was transported down the nutritive tubes to the oocytes. To clarify the first point, ovaries were cultured in medium containing 50µl/ml (5Ci/mM : Radiochemical Centre, Amersham) and ovarioles removed at 2h, 6h, 12h, 24h and 48h, fixed for 5 m in ice cold 5% TCA, digested with NCS (Nuclear Chicago) (0.4 mls NCS : 0.03 mls water/ovariole), and the counts per minute per ovariole determined by liquid scintillation counting. Ovarioles were found to incorporate ^{3}H uridine linearly into RNA during a 48h period (Fig. 1). Secondly, to determine whether rRNA was made *in vitro*, ovaries were cultured for 24h before being transferred to a medium containing ^{3}H uridine (100µl/ml : 30 Ci/mM). After 6h, the RNA was extracted as per Brown and Littna [6], and centrifuged to equilibrium

on 5 - 20% sucrose gradients (54,000 g at 5°C for 20.5h). 25 drop fractions were collected and their optical density of 260 mμ measured. 50μl of each fraction was dried onto cellulose filters and the cpm determined by liquid scintillation. The radioactivity profile revealed newly synthesized ribosomal RNA (see Fig. 2). Finally, to investigate the movement of RNA *in vitro*, ovaries were put in culture, and allowed to 'acclimate' for 24h, and then transferred to fresh medium, with ^{3}H-uridine (10μl/ml : 5Ci/mM). After incubations of 3h, 12h and 24h, the ovaries were fixed in San felice[7], washed in water, dehydrated in ethanol and embedded in methacrylate. Sections (1μm) were cut, mounted on slides, and autoradiographed (as in Macgregor and Stebbings[1]). We observed a progressive movement of labelled RNA with time, from the trophic cells, into the trophic core and thence down the tubes, as has been reported *in vivo*[1].

The ability to culture successfully the telotrophic ovary expands considerably the usefulness of this organ in the study of the structure, function and assembly of microtubules; and this technique is being employed at present to investigate the effects of colchicine, vinblastine sulphate, and other antimitotic agents on the microtubules and the recovery of the system after removal of such drugs.

H. STEBBINGS

N.A. RATCLIFFE

Department of Zoology,
School of Biological Sciences,
University of Leicester,
Leicester, England.

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Fig. 1.

Incorporation of ^{3}H uridine into RNA of single ovarioles over a period of 48h. Each plot is taken from the average count of 10 ovarioles.

Fig. 2.

Sedimentation pattern of radioactivity of RNA from ovaries cultured for 24h and incubated for a further 6h in ^{3}H uridine •——• represents the O.D. of total RNA. •- - -• represents the radioactive newly synthesised RNA.

- Appendix IV -

Structure of Microtubules:

A Study of Freeze-Etched and Negatively Stained Microtubules from the ovaries of Notonecta

by

H. Stebbings* and J.H.M. Willison

Department of Zoology, Adrian Building, University of Leicester, Leicester, and Department of Botany, University of Nottingham, Nottingham, England.

Running head: Freeze-etched microtubules.

*Present address: Department of Biology, University of Exeter, Exeter, England.

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INTRODUCTION:

Cytoplasmic microtubules have been observed in a wide variety of animal and plant cells (Porter, 1966). In the majority of cases, the tissue has been pre-fixed with glutaraldehyde prior to electron microscopy, and after this fixation procedure the morphology of the microtubules has been found to be extremely uniform. In transverse section, microtubules appear as filaments with a central region of low density. Although the diameter of microtubules in different reports varies from 18 - 25nm, the differences in dimensions may be due to differences in measuring techniques, since within a particular cell or tissue, the microtubules are usually the same size. In longitudinal section, microtubules sometimes appear straight and sometimes wavy, and up to several microns in length.

Some information regarding the sub-structure of microtubules has been gained from the examination of thin sections (Ledbetter & Porter, 1963). However, most of the details of the sub-structure of microtubules have come from studies of negatively stained microtubules, both from disrupted cells (Gall, 1966; Barnicott, 1966; Kiefer, Sakai, Solari & Mazia, 1966) and preparations of flagellae (Grimstone & Klug, 1966; Behnke & Zelander, 1967).

There has been growing interest in the spatial relationship between microtubules and the cytoplasm which surrounds them, especially with regard to the possible function of microtubules. Microtubule to microtubule cross connections have been observed in a number of situations (Grimstone & Cleveland, 1965; Tucker, 1968; Tilney & Byers, 1969; Roth, Pihlaja & Shigenaka, 1970), and it is generally accepted that the cross bridges function to hold the tubules together, thus providing rigidity. In contrast, where microtubules are found in tracts of cytoplasmic flow,

a 'clear zone' has been observed around individual tubules from which other cellular material is excluded (Ledbetter & Porter, 1963; Silver & McKinstry, 1967; Macgregor & Stebbings, 1970). There are a few reports of arms or cross bridges which seem to link microtubules to particles which move alongside them (Pickett-Heaps & Northcote, 1966; Smith, 1971), and Tucker (1972) has described arm bearing microtubules which he suggests are involved in the propulsion of material alongside the tubules.

Since the majority of evidence as to the structure of microtubules and their relationship to other cytoplasmic inclusions comes from observations of fixed material, the question arises, how does this observed structure relate to the structure of microtubules in the living cell?

One of the most extensive aggregations of microtubules yet observed, is that in the telotrophic ovaries of Hemipteran insects. Macgregor & Stebbings (1970) have found that in the ovary of Notonecta, the trophic tubes which link an anterior trophic region to a string of developing oocytes, each contain approximately 30,000 microtubules; and that ribosomes pass along the trophic tubes to the oocytes, where they are stored.

In this study we have employed the freeze-etch technique to look at the system of microtubules in the ovary of Notonecta, and have compared these observations with those from negatively stained microtubules isolated from these ovaries. In this way, we hoped to observe microtubules which had not been subjected to any chemical fixation, dehydration or embedding processes and relate these observations to the structure and arrangement of microtubules seen in fixed material.

MATERIALS AND METHODS:

Notonecta glauca glauca Linn. were obtained from a fire pond in Tentsmuir Forest, Fife, Scotland.

Initial attempts to isolate and negatively stain microtubules from the ovary of Notonecta proved that they are extremely labile structures. It was therefore essential to stabilise the microtubules with buffered hexylene glycol in conjunction with cold, and a slightly acidic pH - a technique first described by Kane (1962) and more recently modified by Kirkpatrick (1969). Ovaries were removed, and the portions containing the smallest oocytes and the most trophic tubes, the 'neck' region, were excised from two ovaries and lightly homogenised in buffered hexylene glycol on ice (1M hexylene glycol in 0.003M potassium buffer, pH 6.5). Material was picked up on Formvar/carbon coated grids and negatively stained with uranyl acetate and phosphotungstic acid (as per Gall, 1966).

For freeze-etching, ovaries were removed and small pieces of ovarioles placed in 20% glycerol in Ringer's solution for 10m. The 'neck' region was then excised and mounted on collared gold specimen holders before freezing in Freon 22. The freeze-etching method was according to the now standard technique of Moor & Muhlethaler (1963). The etched specimens were shadowed with platinum/carbon from an electron beam source in a 'Balzers' 360M freeze etcher. The replicas of the frozen fractured surfaces were cleaned using commercial bleach (Chloros, ICI) for 3h followed by 70% sulphuric acid for 6h, and then mounted on Formvar coated grids.

The negatively stained preparations, and freeze-etch replicas were examined using AEI EM 6B and 802 electron microscopes.

OBSERVATIONS:

After isolation in buffered hexylene glycol, followed by negative staining, lengths of microtubules could be seen over the surfaces of the grids. The microtubules were straight or slightly curved, and broken ends could be seen. Where individual microtubules were free from other material, they were more collapsed (Fig. 1), and in some cases even split open (Fig.2). In such split microtubules, 11 parallel sub-filaments were visible in the wall (Fig. 2) and repeating sub-units of approximately 4nm were measured along the length of many sub-filaments.

Microtubules were visible in freeze-etch replicas of trophic tubes (Figs,3,4). In longitudinal fractures, the microtubules were straight, almost parallel and rarely touched each other. The surfaces of certain microtubules were exposed by the fracture process and others were split open to reveal the central 'core' (Fig.5). In some cases both outer and internal surfaces of single microtubules were revealed. At the internal surface especially, a longitudinal repeat of 4nm was sometimes visible, which corresponds with the negative-stained image (Fig.3).

Oblique and transverse fractures of trophic tubes showed the circular profiles of microtubules (Figs.4,6). The microtubules were approximately 26nm in diameter, compared to 20nm diameter of fixed and sectioned microtubules (Macgregor & Stebbings, 1970). In both oblique and longitudinal fractures (Figs.5,6), parallel sub-filaments could be seen in part of the wall of some microtubules.

The negatively stained preparations show no structural projections from the microtubules. Amongst the tubules were round structures, which we assume to be ribosomes; because of their size and because microtubules and ribosomes are the only visible components seen in thin sections of

the trophic tubes (Macgregor & Stebbings, 1970). Although there were ribosomes between the microtubules, in some regions, the surfaces of the microtubules appear to be 'encrusted' with ribosomes.

In the freeze-etch longitudinal fractures, hairy projections can be seen from individual microtubules and more obviously between adjacent microtubules (Figs 3,7). These projections ranged from 10 - 20nm in length.

In transverse fracture both the microtubule 'core' and the 'clear zone' which surrounds each microtubule, can be seen. This arises as a result of ice formation in these regions during freezing and the subsequent etching of this ice.

DISCUSSION:

The principal objective of this study was to compare the appearance of the microtubules in freeze-etch replicas of trophic tubes, with that of microtubules in fixed and sectioned material, and also with microtubules which had been isolated from whole ovaries and negatively stained. Some differences were immediately apparent. In longitudinal sections of trophic tubes, the microtubules have been described as finely wavy (Macgregor & Stebbings, 1970) whereas in both the freeze-etch replicas and the negatively stained preparations, the microtubules appear straight or only gently curved. We regard the straightness to be closer to the true situation, since there is less chance of a change occurring with freeze-etching, and it seems less likely that wavy microtubules would straighten than vice-versa. Jensen and Bajer (1969) have attributed a corresponding waviness of spindle microtubules, in the dividing endosperm cells of Haemanthus, to a post-mortem shrinkage of adjacent long chromosomes.

The information regarding the sub-structure of microtubules which was obtained from the examination of freeze-etch replicas corresponds in many respects with that gained from studies in which the microtubules had been subjected to chemical fixation. Sub-filaments have been observed in the walls of microtubules after thin sectioning (Ledbetter & Porter, 1963) and after negative staining (Andre & Thiery, 1963; Kieffer, Sakai, Solari & Mazia, 1966; Barnicott, 1966). In our oblique and longitudinal fractures, sub-filaments could be seen in a sector of the walls of many microtubules. There has been speculation as to the number of sub-filaments within the walls of microtubules, but the freeze-etch technique did not resolve this question, since the sub-filaments were never visible all the

way round a single microtubule. Ledbetter & Porter (1964) used the photographic rotational reinforcement technique of Markham, Frey and Hills (1963) to reinforce the image of plant microtubules in thin transverse sections, and concluded that there were probably 13 sub-filaments within the walls of the microtubules which they examined. Numerous studies of negatively stained microtubules have reported different numbers of sub-filaments in the walls of the tubules. These figures vary from 10 - 14 (see Behnke & Zelander, 1967), and it is quite possible that microtubules in different situations are composed of different numbers of sub-filaments. In our negatively stained preparations, where individual microtubules had split open and presumably unfolded onto the grids, 11 parallel sub-filaments were counted.

Using the freeze-etch technique, Northcote and Lewis (1968) have observed microtubules beneath the plasmalemma at the 'cytoplasmic surface' of pea root tip cells and recorded that the tubules showed a distinct sub-structure along their length, the nature of which was not discernible with the resolution they were able to achieve. Using the same technique, to examine yeast cells, Moor (1967) has proposed a microtubule model with the walls consisting of 4nm sub-units arranged in a double helix, and the sub-units aligned in parallel rows, corresponding to the beaded sub-filaments seen after negative staining. Our preparations also showed a periodicity of 4nm along many fractured microtubules, especially where the fracture had occurred through the 'cores' of the microtubules. Although care must be taken in interpreting the reality of apparent sub-structure close to the limit of resolution of the freeze-etch technique (Willison & Cooking, 1972), we regard the periodicity seen along the microtubules in freeze-etch replicas as real, since it matches closely that seen along

microtubules which have been negatively stained.

A number of other points emerge from our freeze-etch study. In longitudinal fractures of trophic tubes, the microtubules sometimes fractured to reveal their surfaces and in other cases their internal structure. Willison and Cooking (1972) have found a similar occurrence when rod-like viruses are fractured.

What is the significance of the projections from, and the distinct bridges between microtubules, in the freeze-etch preparations? There are a few reports where arms or cross bridges seem to link microtubules to particles which move alongside them. Pickett-Heaps and Northcote (1966) have shown bridges which link vesicles of the smooth endoplasmic reticulum to spindle tubules in wheat meristem cells, and Smith (1971) has described bridges which join synaptic vesicles to neurotubules in the axons of the lamprey. Tucker (1972) has observed rows of arm-bearing microtubules lining the sucking mouthparts of ciliates and has suggested that the arms are involved in the propulsion of material alongside the tubules.

Macgregor and Stebbings (1970) have shown that ribosomes pass along the trophic tubes which are packed with cytoplasmic microtubules, but they did not observe any prominent arms or cross bridges associated with the microtubules in their thin sections after conventional fixation and staining techniques. The bridge-like structures between the microtubules and the projections from individual tubules seen in the freeze-etch replicas, may be functionally important, while on the other hand, they may be simply eutectic structures, arising as a result of ice crystals growing between the microtubules.

A 'clear zone' has been described around individual microtubules (Ledbetter & Porter, 1963; Silver & McKinstry, 1967; Macgregor & Stebbings, 1970), and it has been suggested that this 'clear zone' may be the result

of shrinkage on fixation (Maser & Philpott, 1964). However, Lane & Treherne (1970) have found that if axons from cockroach ganglia were fixed and stained with lanthanum hydroxide, as opposed to the usual preparative techniques, then the 'clear zones' around the tubules stained as did their cores. They concluded that the 'clear zones' were real structures, probably composed of mucopolysaccharide. Substantially similar structural features are seen in transverse and oblique fractures of microtubules, in that their walls are dark and the zone around individual tubules, and their cores, appear light in shadowed replicas.

Although the freeze-etch technique does not provide any evidence as to the composition of the 'clear zone', except that it probably has a high water content, our observations confirm that they are real structures, and not artifacts of fixation. Indeed, some authors have stressed that microtubules may have structural components not shown by conventional fixation and staining techniques (Silver & McKinstry, 1967; Forer, 1969).

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LEGENDS TO FIGURES

Fig. 1.

Microtubules isolated from ovaries of Notonecta in hexylene glycol and negatively stained. Where individual microtubules are free from ribosomes they are more collapsed and show sub-filaments in their walls and repeating sub-units along their lengths.

Fig. 2.

This figure shows a microtubule which has split open and unfolded onto the grid (see arrows) revealing all the sub-filaments which comprise the wall of the tubule.

Fig. 3.

Longitudinally fractured microtubules in a frozen-etched trophic tube. The microtubules appear straight and a periodicity of approximately 4nm can be seen along parts of some of the tubules. In many places, projections can be seen from the microtubules.

Fig. 4.

Transversely fractured microtubules within a frozen-etched trophic tube. With this technique, the 'cores' of the microtubules etch to the same extent as the 'clear zones' which surround individual microtubules.

Fig. 5.

Longitudinally fractured microtubules in a frozen-etched trophic tube. The microtubules fracture both at their surfaces (arrowed) and internally, revealing their tubular structure. The outer surfaces of the microtubules display longitudinal stiations which correspond with sub-filaments.

Fig. 6.

Obliquely fractured microtubules in a frozen-etched trophic tube. The arrangements of sub-filaments in the wall of the microtubules are particularly clear in this case (arrows).

Fig. 7.

Longitudinally fractured microtubules in a frozen-etched trophic tube. Apparent cross-bridges between closely adjacent microtubules are commonly found. This effect might be a technical artifact.

RNA SYNTHESIS, MOVEMENT, AND CYTOPLASMIC MICROTUBULES, IN THE TELOTROPHIC OVARY OF NOTONECTA GLAUCA GLAUCA (Linn.)

A Thesis presented for the degree of

Doctor of Philosophy to the University of St. Andrews

by Howard Stebbings B. Sc.

Department of Zoology, The University of St. Andrews,

St. Andrews, Fife, Scotland

and

Department of Zoology, University of Leicester, Leicester,

England.

VOLUME II

TEXT FIGURES

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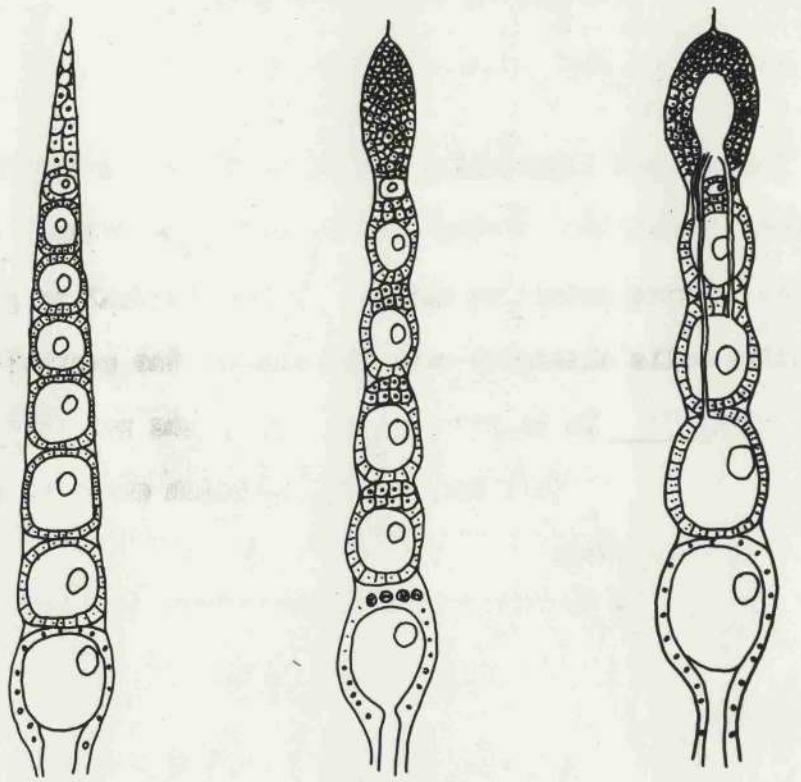
Fig. 1.

This figure illustrates the three different types of insect ovaries. Panoistic ovaries have no nutritive cells. Meroistic ovaries do have nutritive cells. When individual or groups of nutritive cells alternate with the oocytes the ovariole is known as polytrophic. In telotrophic ovarioles the nutritive cells are confined to an anterior trophic region which connects to each oocyte by a nutritive tube.

THREE TYPES OF INSECT OVARIOLE.



PANOISTIC

POLYTROPHIC

TELOTROPHIC

MEROISTIC

Figs. 2,3 and 4 are sections stained with gallocyanine-chrome alum.

Fig. 2.

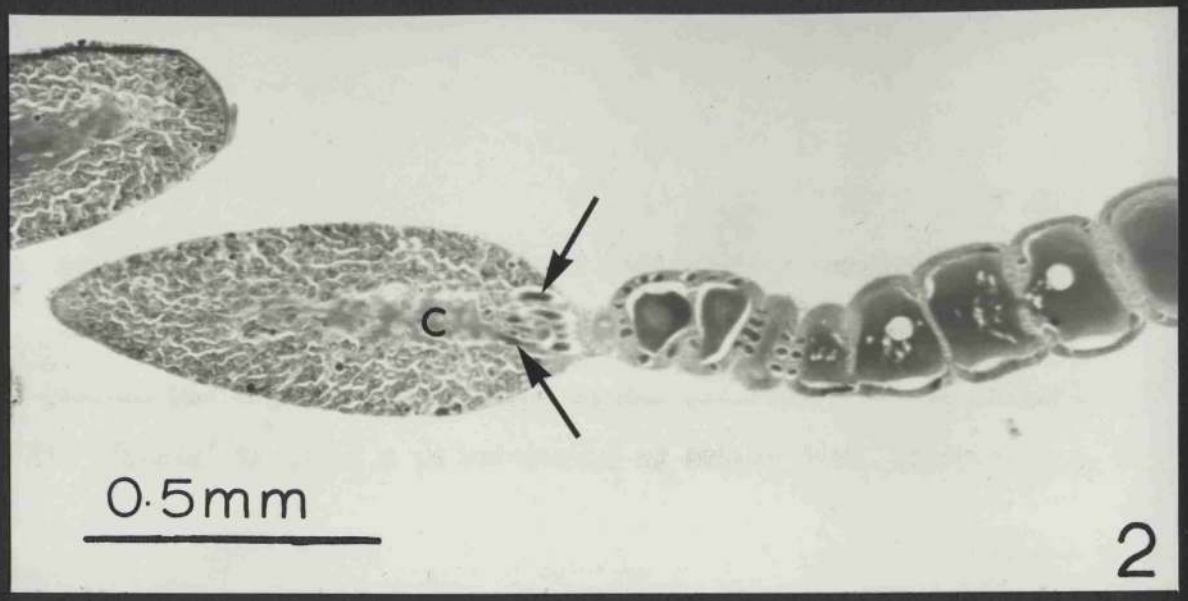
Longitudinal section (LS) through an ovariole of Notonecta. Nutritive cells are arranged around the trophic core (c). Small lengths of tube (arrowed) can be seen passing back to the developing oocytes. Each oocyte is surrounded by a layer of follicle cells.

Fig. 3.

Trophic region. Branches of the trophic core (c) interdigitate between the nutritive cells. The nutritive tubes (arrowed) pass back from the trophic core, around the prefollicular region, which contains young oocytes.

Fig. 4.

L.S. through part of a nutritive tube showing 'hair-like' wavy striations.



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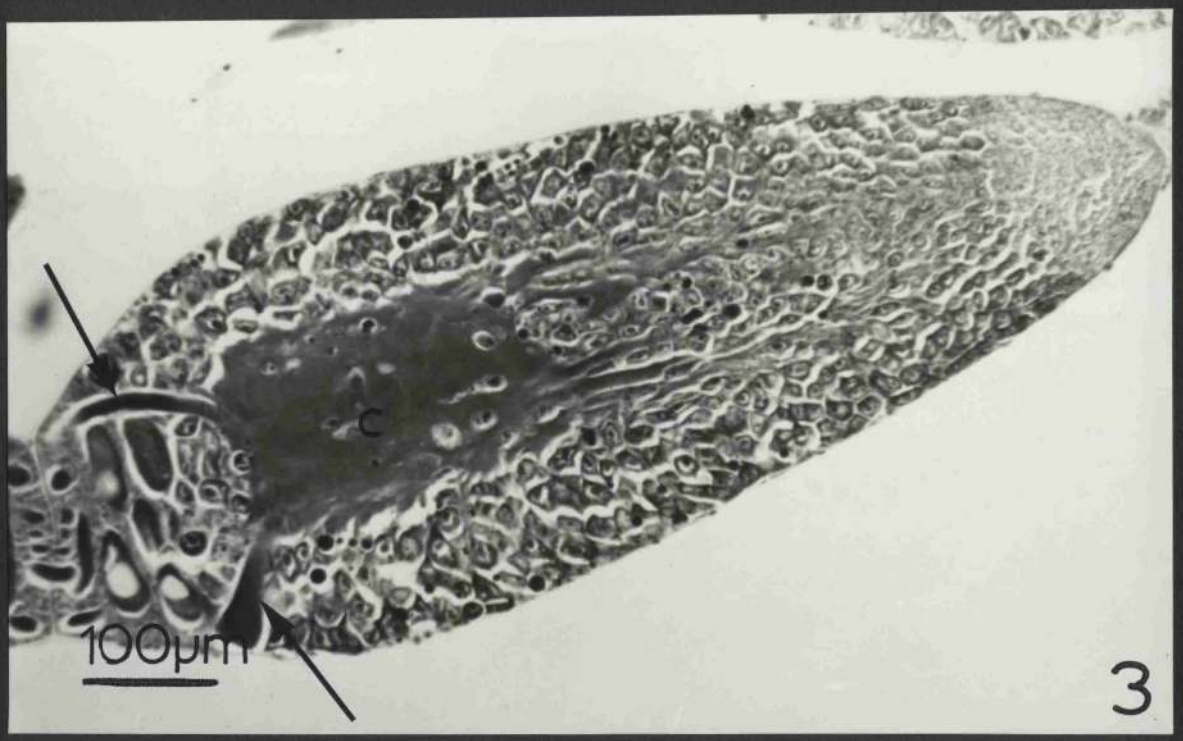

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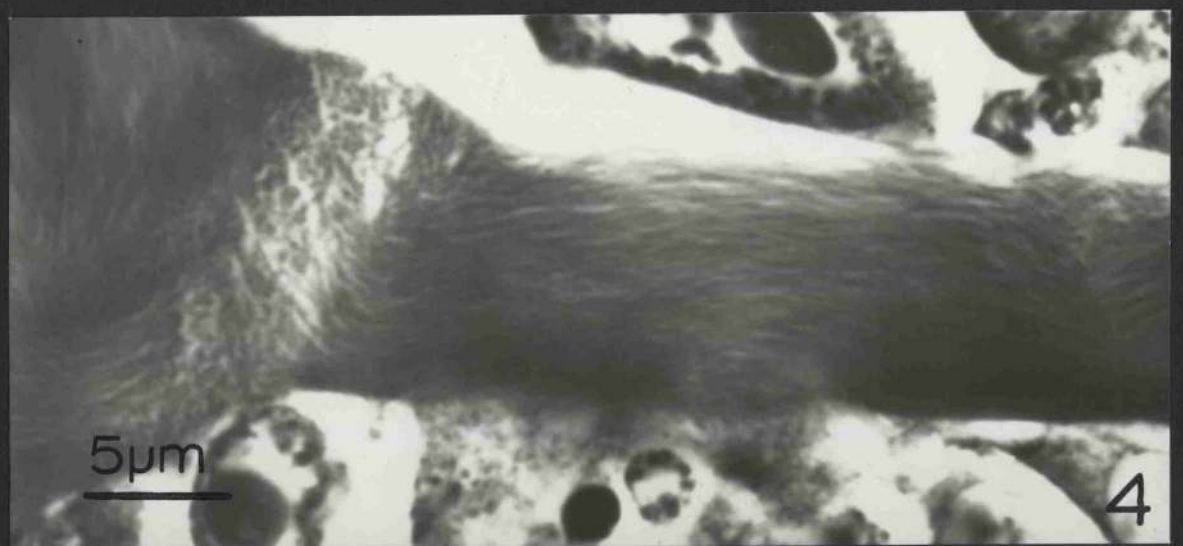


4

Figs. 5,6 and 7 are Feulgen stained sections.

Fig. 5.

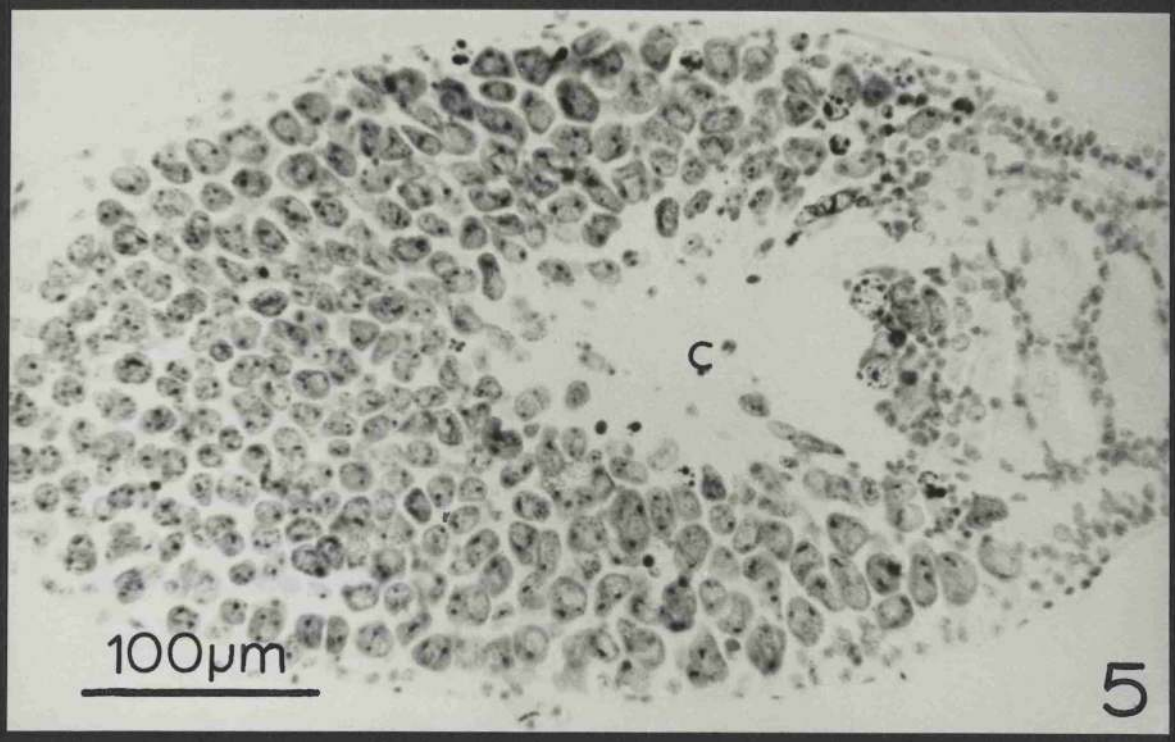
The trophic core (c) and nutritive tubes are Feulgen-negative.

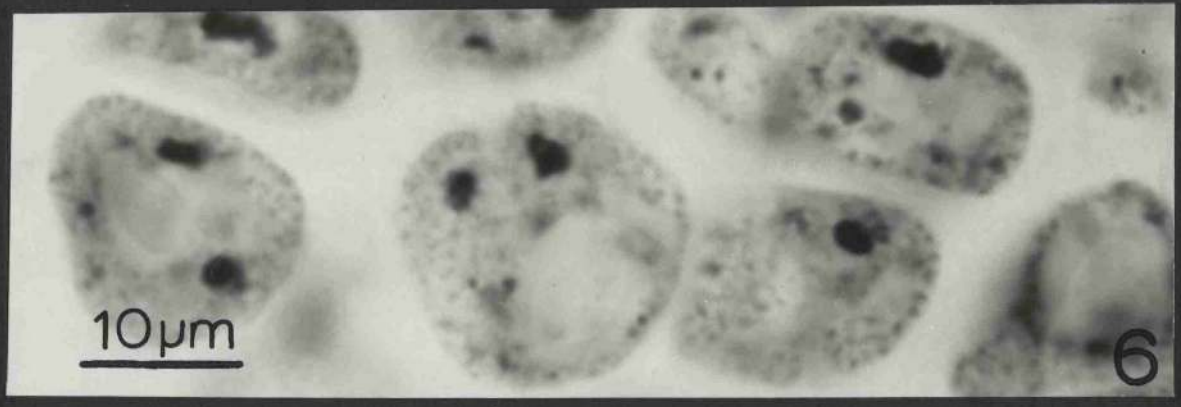
Fig. 6.

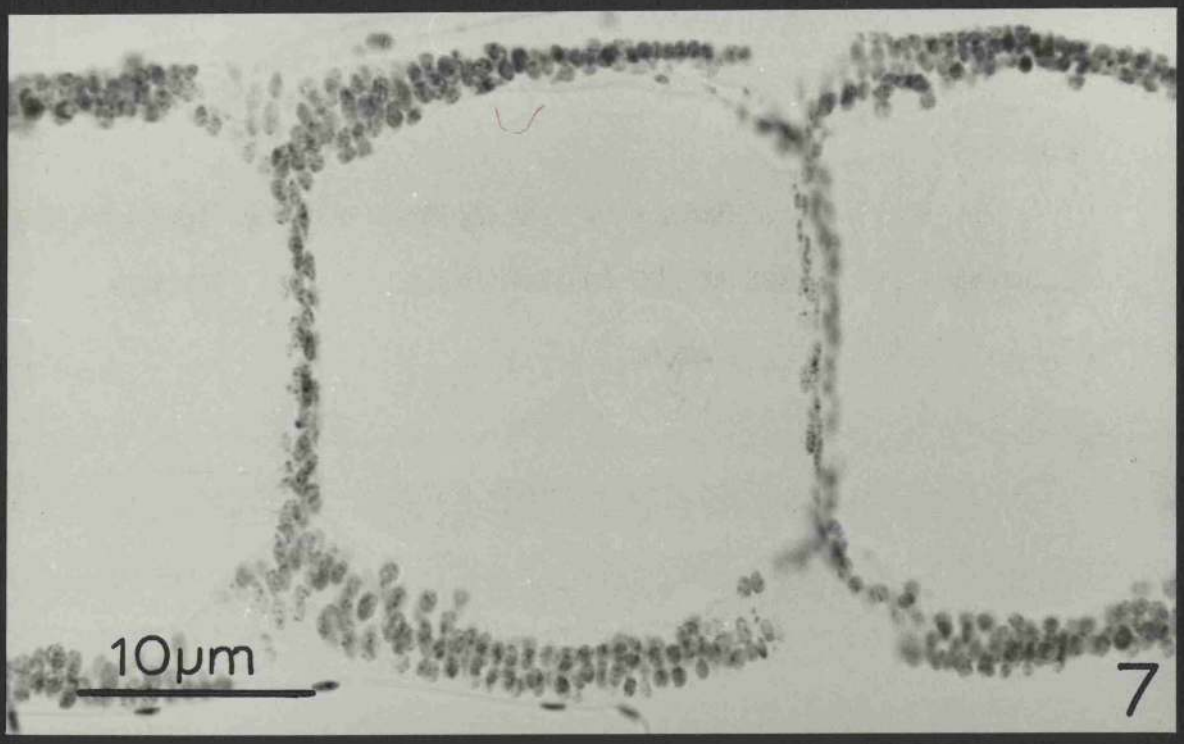
The largest trophic nuclei have two or three Feulgen-positive patches around a Feulgen-negative nucleolus.

Fig. 7.

Oocyte nuclei contain only faintly detectable Feulgen-positive material. The nuclei of the follicle cells stained strongly.


ç
100µm
5


10µm
6


10µm
7

Figs.8 - 11 are Feulgen stained squash preparations of nutritive cells.

Fig. 8.

Squash preparation of large nutritive cells showing two characteristic heterochromatic patches.

Fig. 9.

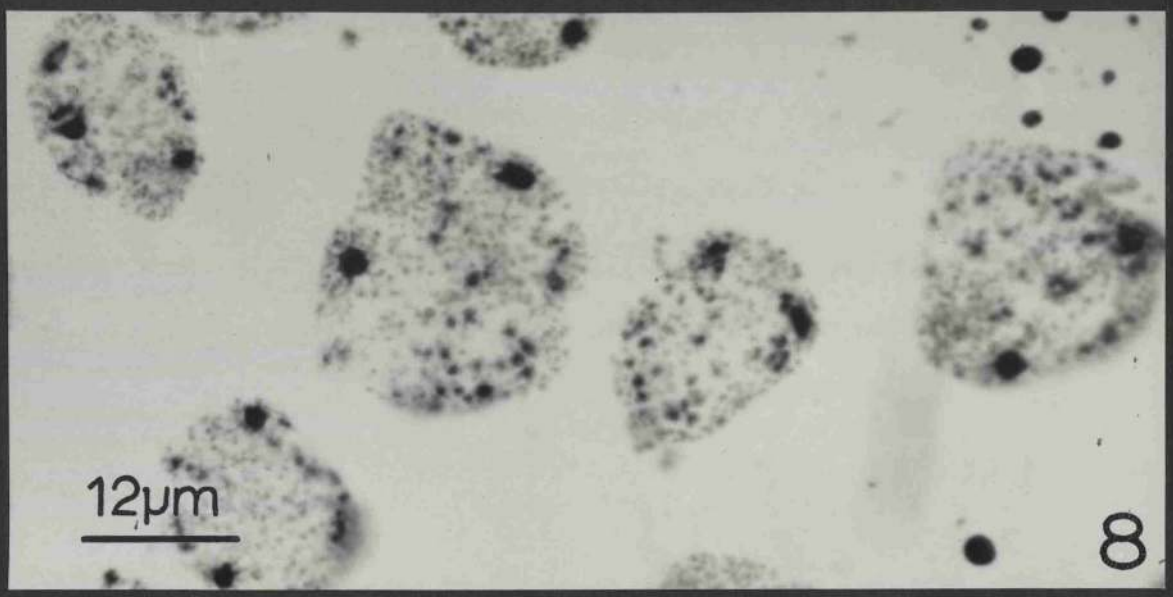
With slightly greater pressure, the nuclear membrane ruptures and the heterochromatic masses are themselves compressed.

Fig. 10.

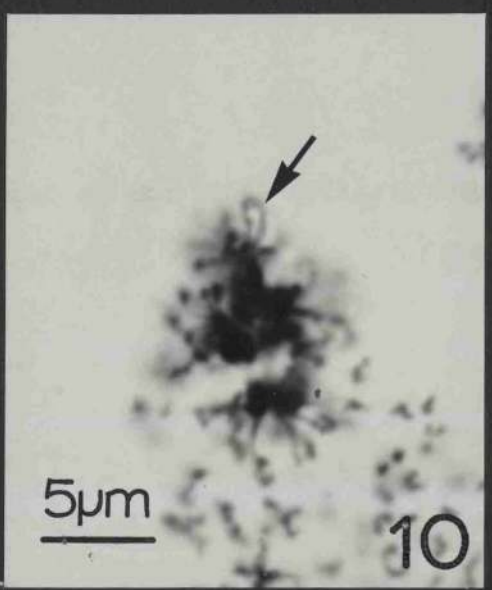
In some squash preparations Feulgen-positive loops can be seen from the heterochromatic masses (arrowed)

Fig. 11.

Loops from the heterochromatin vary in length.


12µm
8

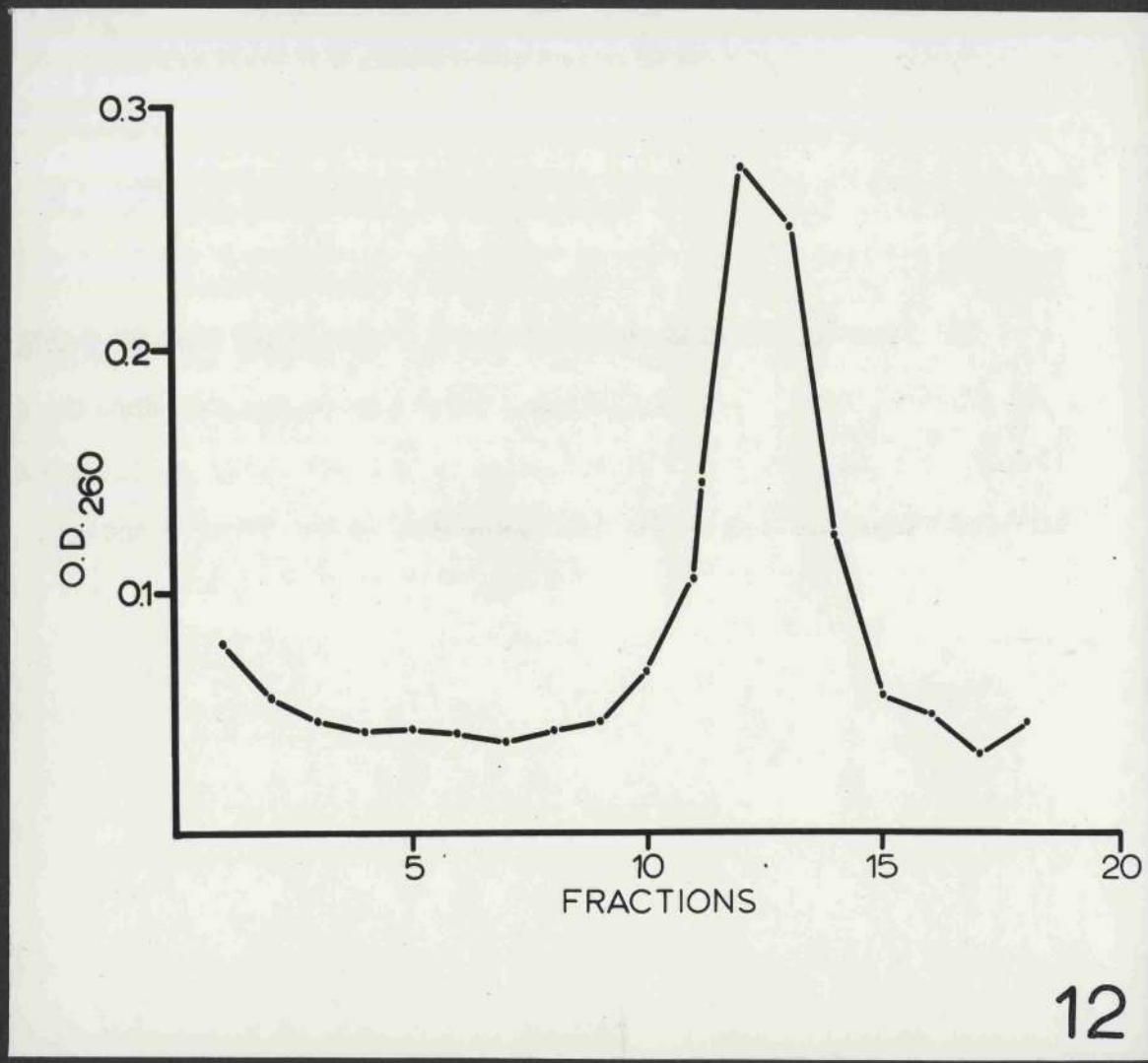

12 µm
9


5µm
10


5µm
11

Fig. 12.

DNA from the trophic region forms a single band when centrifuged to equilibrium on a CsCl gradient. There is no separate band of high density DNA such as would be expected if the nucleolar or ribosomal DNA had undergone a specific amplification in the trophic nuclei.



12

Figs. 13,14, 15 and 16 are autoradiographs made after ^{3}H-uridine incorporation, stained with methylene blue.

Fig. 13.

Autoradiograph made 24h after injection of ^{3}H-uridine. There is heavy labelling over the nucleoli of the nutritive cells, over the trophic core (c) and over the nutritive tubes (arrowed). The nuclei of the oocytes are scarcely labelled at all.

Fig. 14.

After 24h incorporation, the nutritive tubes (arrowed) are heavily labelled. The oocyte cytoplasm and the germinal vesicle are lightly labelled.


C
50µm
13

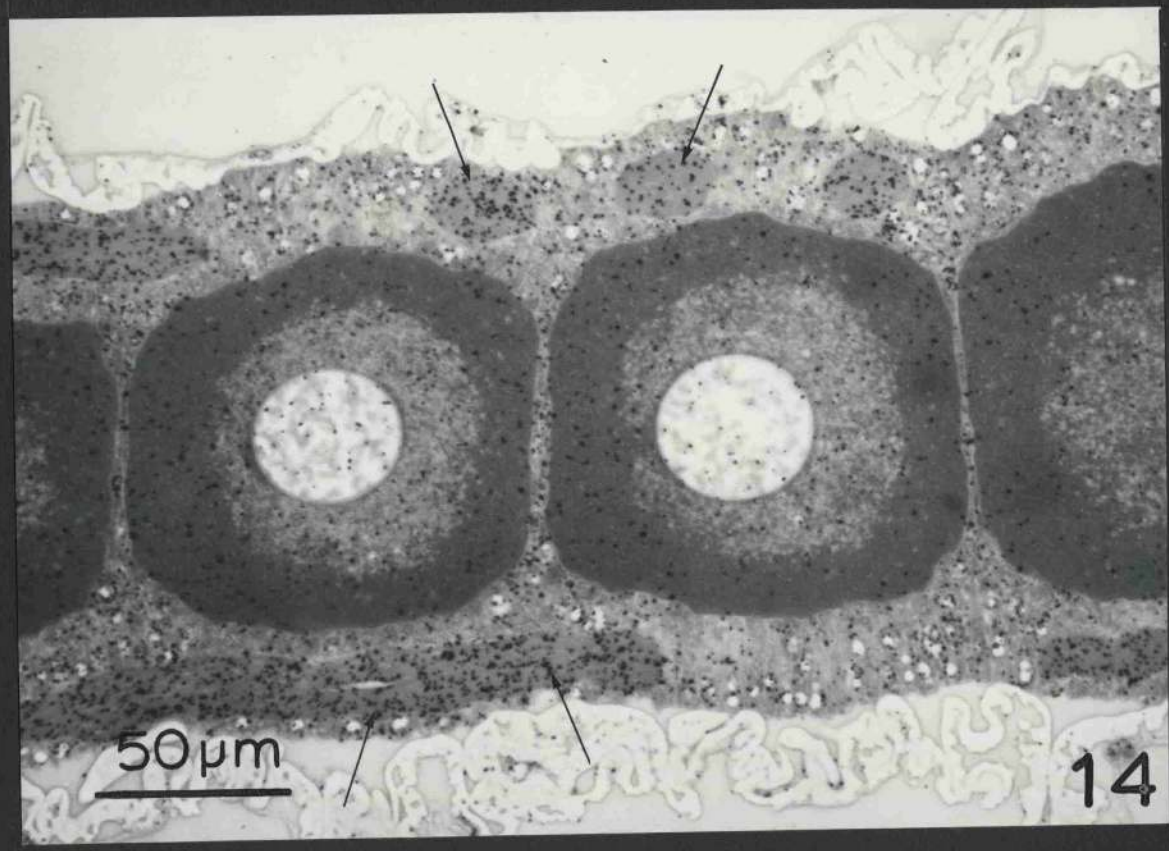
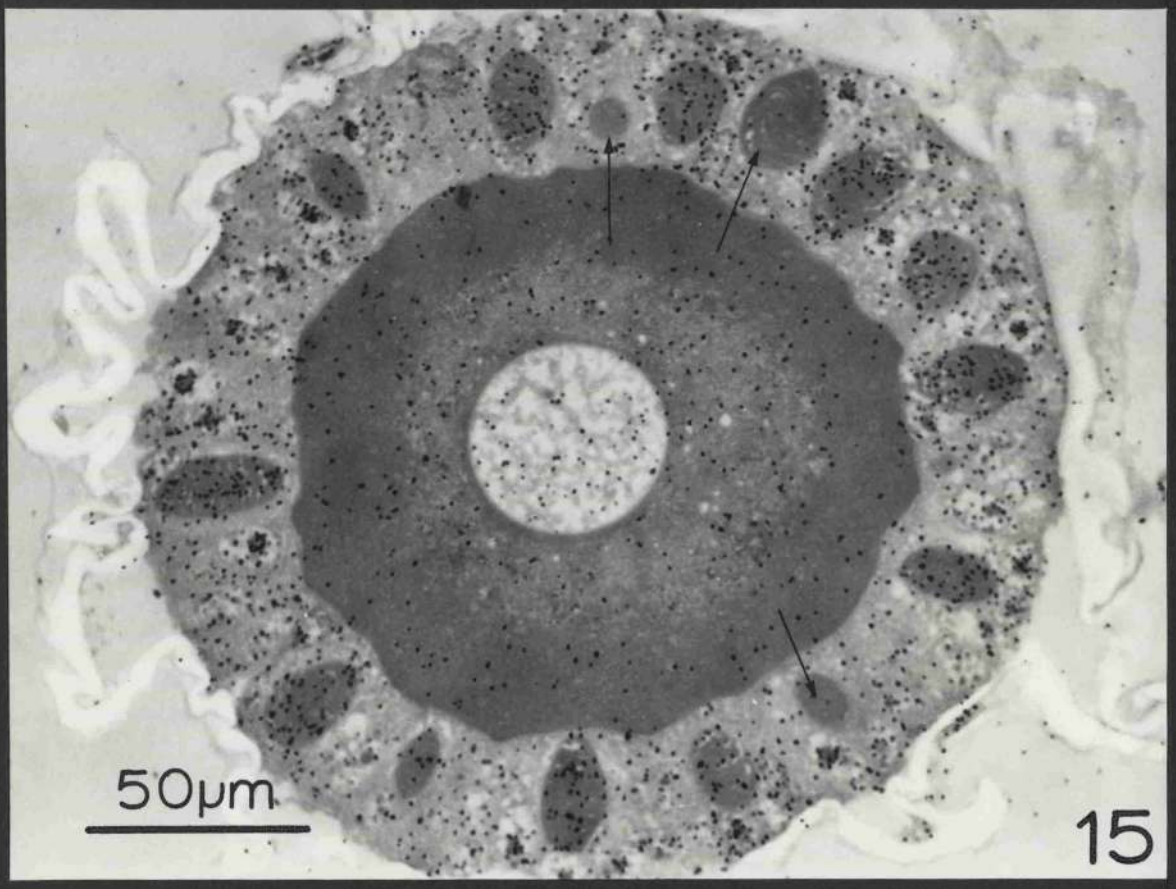

50µm
14

Fig. 15.

Autoradiograph made 24h after injection of ^{3}H-uridine. In this transverse section most of the nutritive tubes are similarly labelled, but three tubes are quite unlabelled (arrowed).

Fig.16.

This autoradiograph shows an almost 'cold' nutritive tube bounded by 'hot' follicle cells, indicating that there is little movement of RNA into the nutritive tubes from the surrounding follicle tissue.


50µm
15

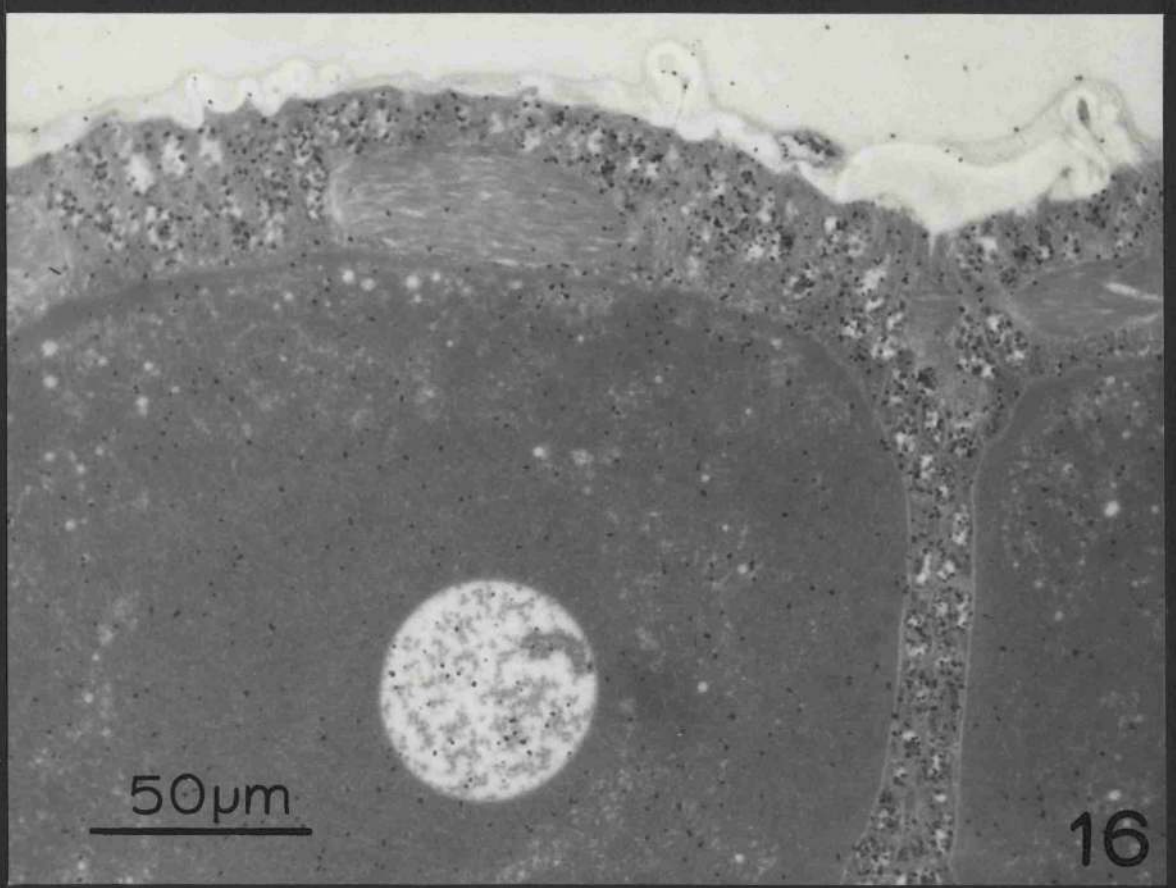

50µm
16

Fig. 17.

Histograms of grain counts in different regions of ovarioles fixed at different times after injection of ^{3}H-uridine. Grain counts are given in full in Table 3.

Number of grains over an area of 150μ^2 in two regions of the the trophic core, regions of tubes alongside successive follicles and cytoplasm of the 7th follicle, in preparations fixed at different times after ^{3}H-uridine injection.

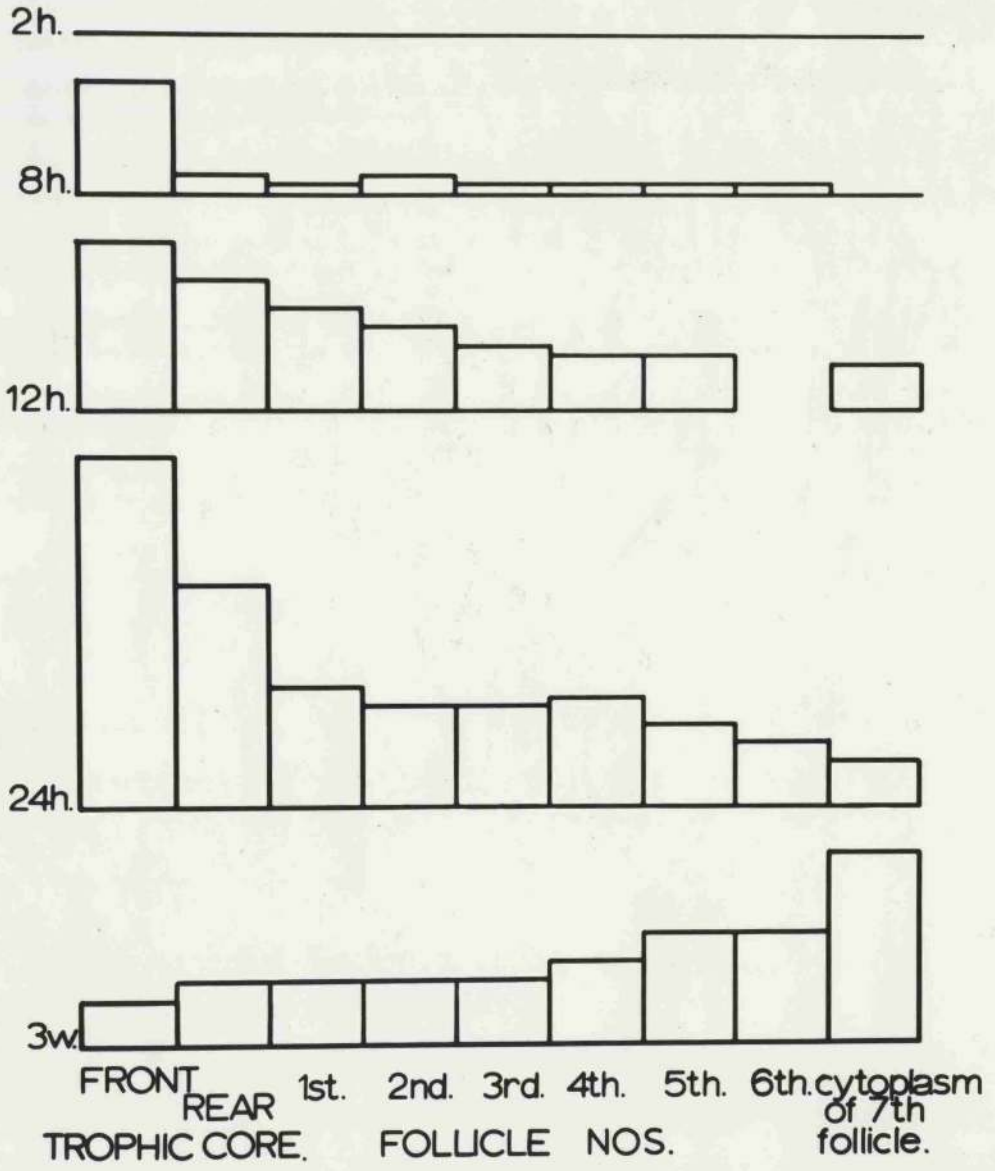


Fig. 18.

Squash preparation of large trophic nuclei to which has been annealed a synthetic radioactive ribosomal RNA, prior to autoradiography. Giemsa stained. The silver grains are evenly distributed indicating that the ribosomal genes are dispersed throughout these polyploid nuclei.

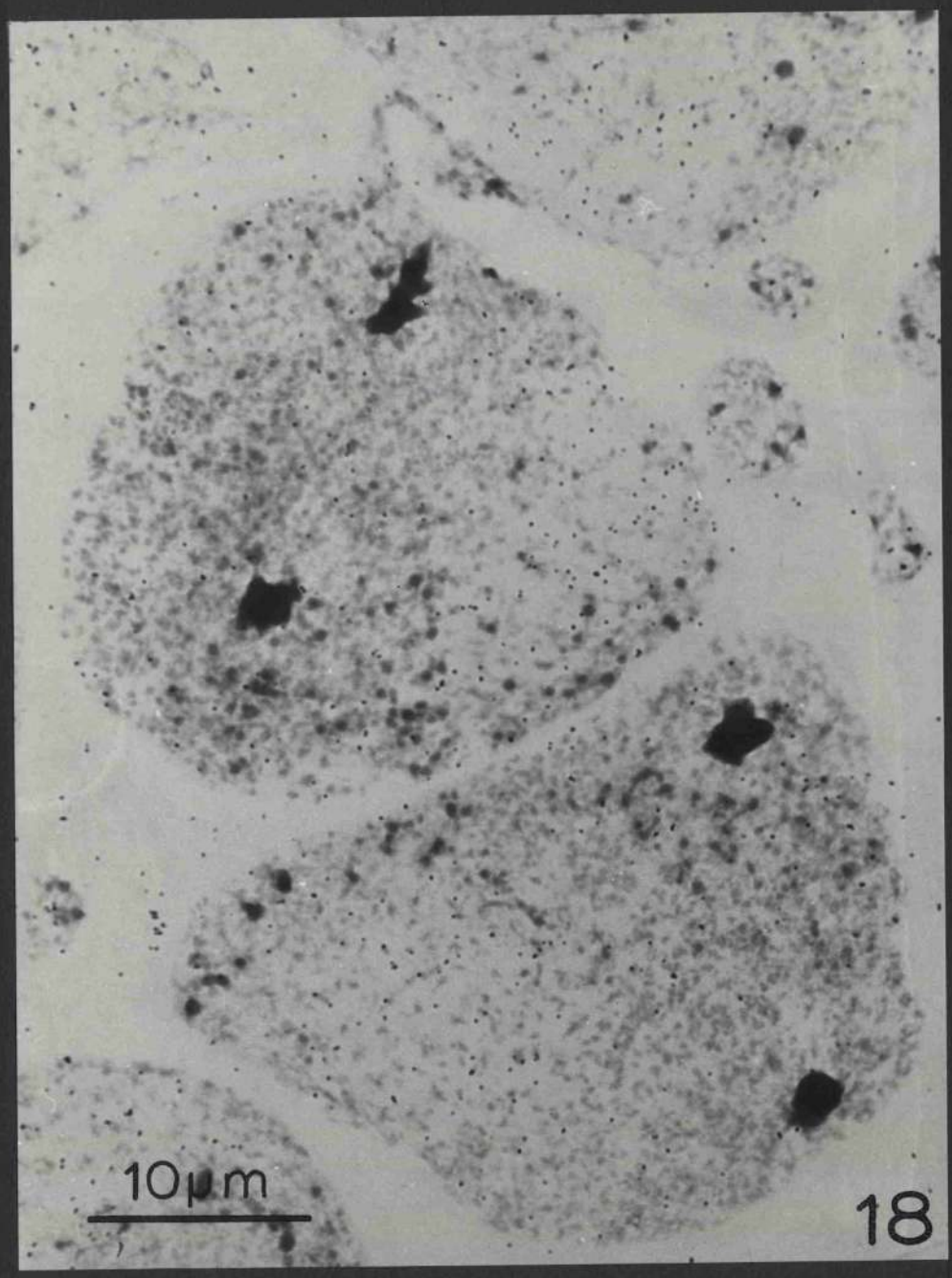
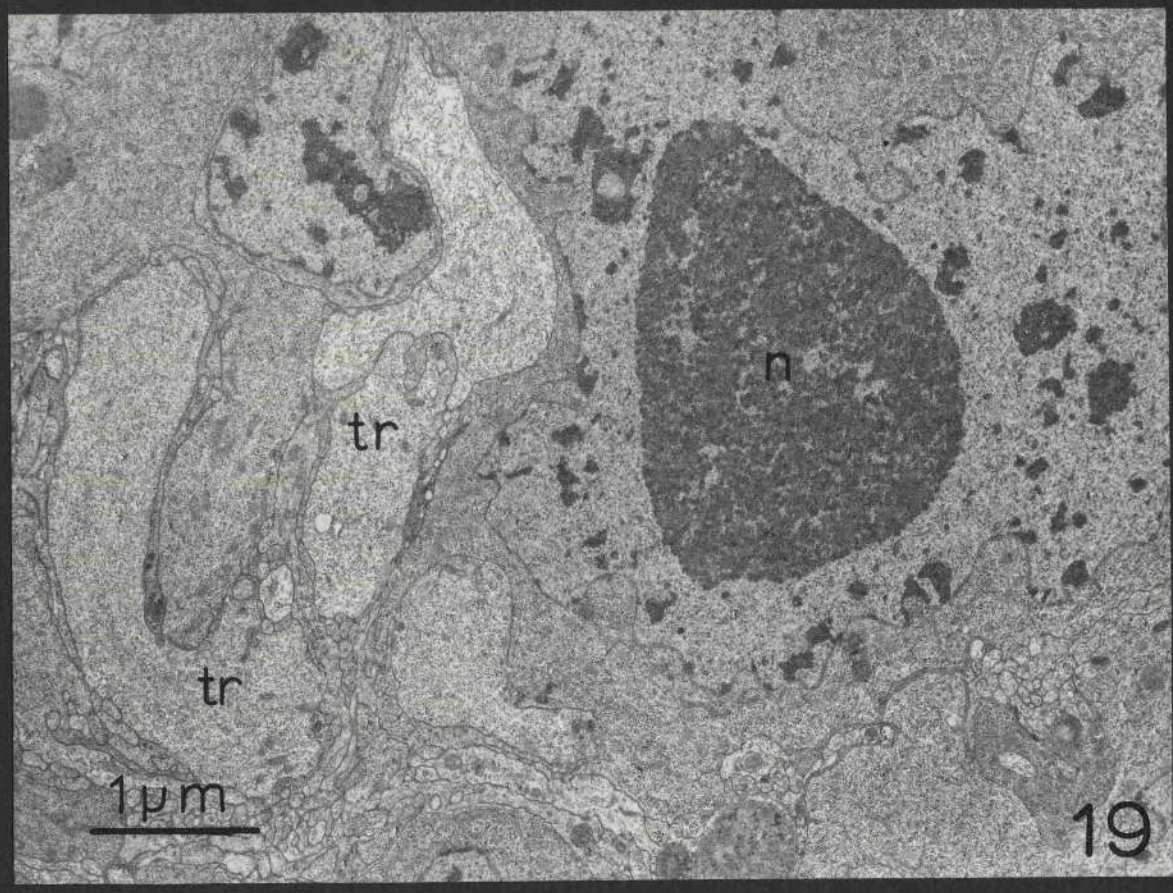

10µm
18

Fig. 19.

Electron micrograph of part of the trophic region of an ovariole. Trophic nuclei have large nucleoli (n) surrounded by chromosomal material. Cell membranes are incomplete and much of the trophic tissue is syncitial. There are tracts (tr) among the trophic nuclei and these are packed with ribosomes.

Fig. 20.

The trophic core (c) is packed with ribosomes and microtubules. This electron micrograph shows flow patterns of ribosomes and microtubules (arrowed) around a trophic nucleus at the edge of the trophic core.


n
tr
tr
1µm
19

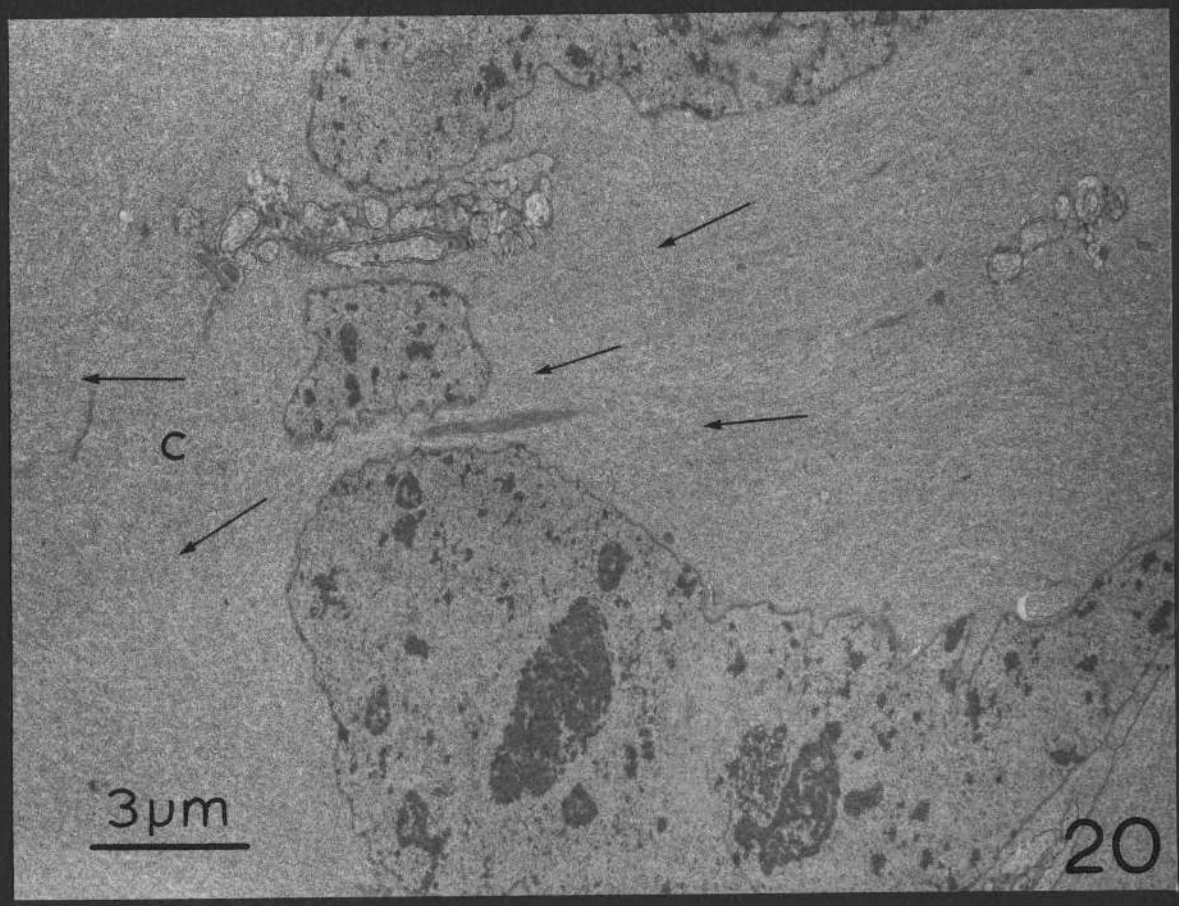

c
3µm
20

Fig. 21.

Electron micrograph of an irregular shaped trophic nucleus at the edge of the trophic core. The trophic core is packed with ribosomes and microtubules and some scattered mitochondria.

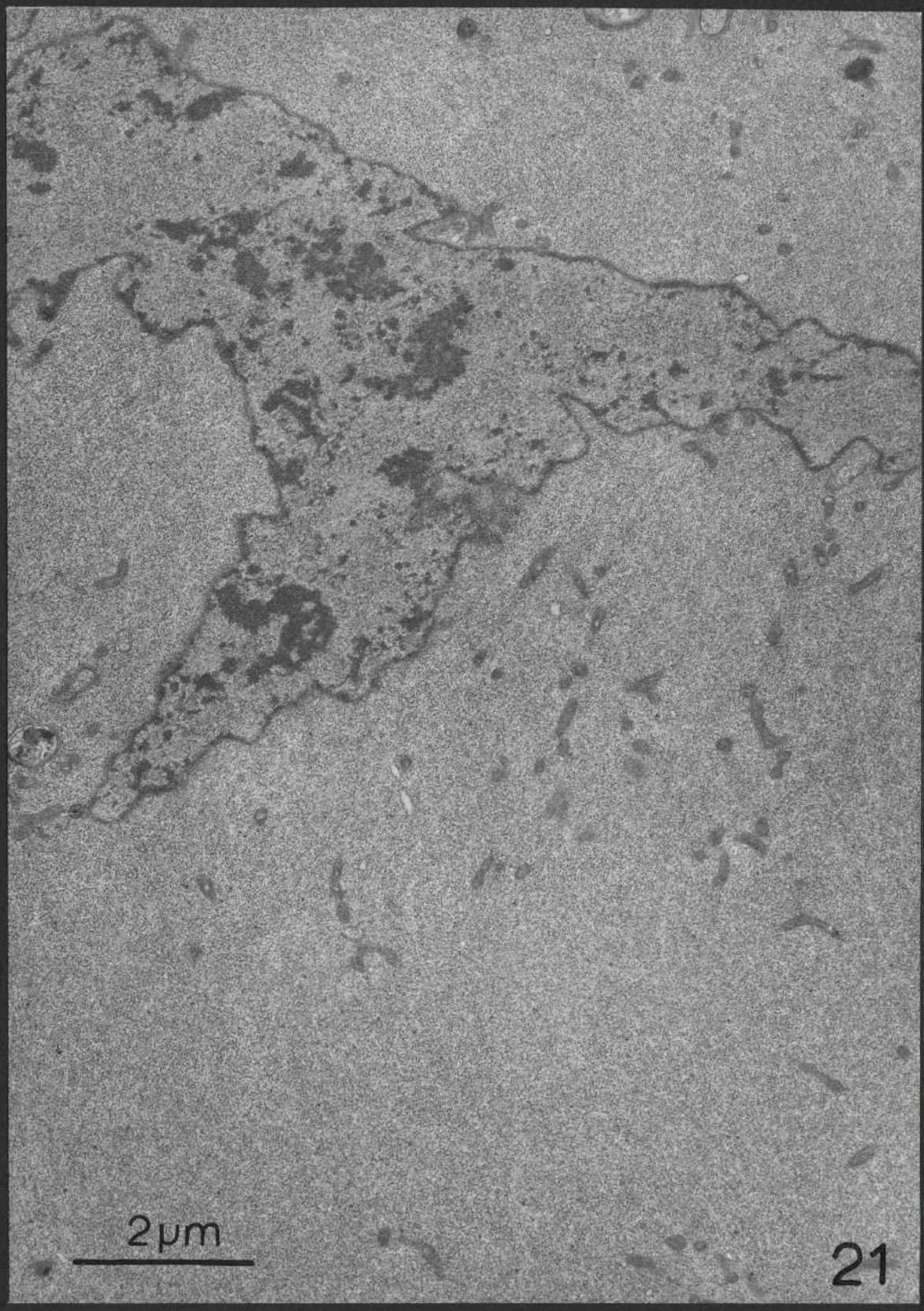

2µm
21

Fig. 22.

Longitudinal section of a nutritive tube. Parallel microtubules can be seen along the length of the tube. Microtubule lengths of up to 2μm can be traced in this micrograph. At the apex of the inpushing into the tube, microtubules are compressed together (arrowed), while on either side of the inpushing there is an absence of microtubules. This suggests that the microtubules are longer than the lengths seen in this micrograph.

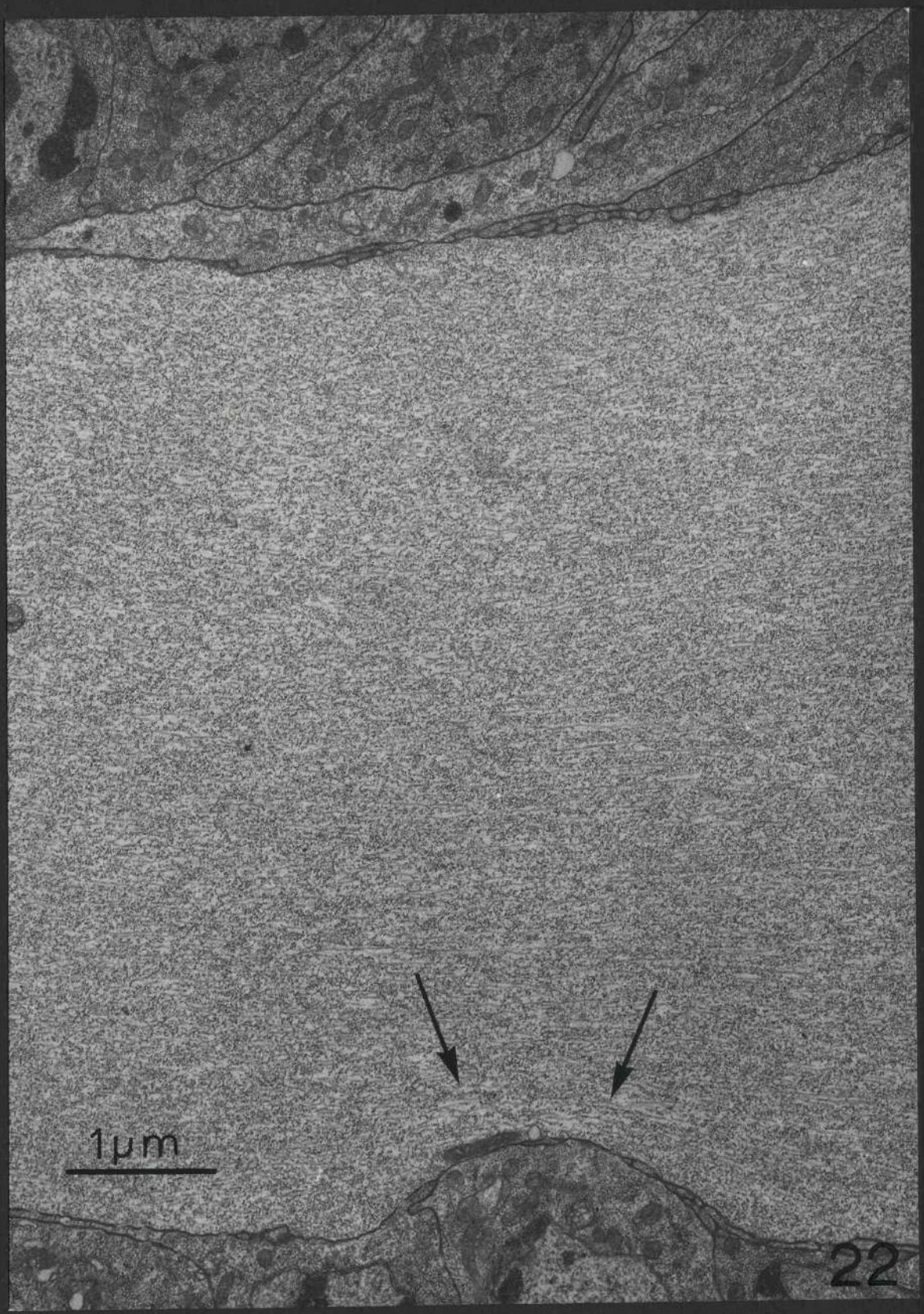
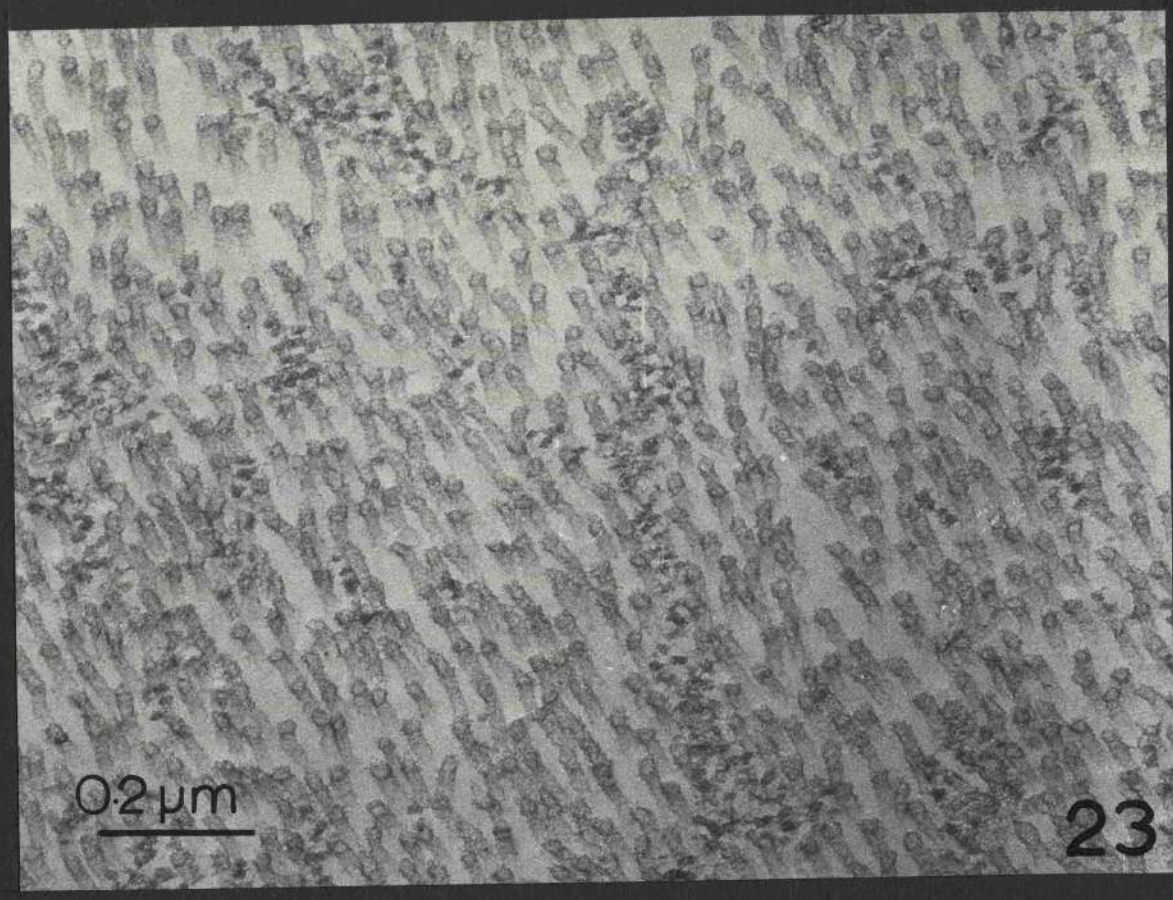

1µm
22

Fig. 23.

Oblique section through a nutritive tube. Microtubules are closely aligned with bands of ribosomes between some microtubules.

Fig. 24.

Longitudinal section through a nutritive tube. Where there is a close alignment of microtubules the cytoplasm is relatively free from ribosomes. Ribosomes can be seen in bands between the aggregations of microtubules.


0·2 µm
23

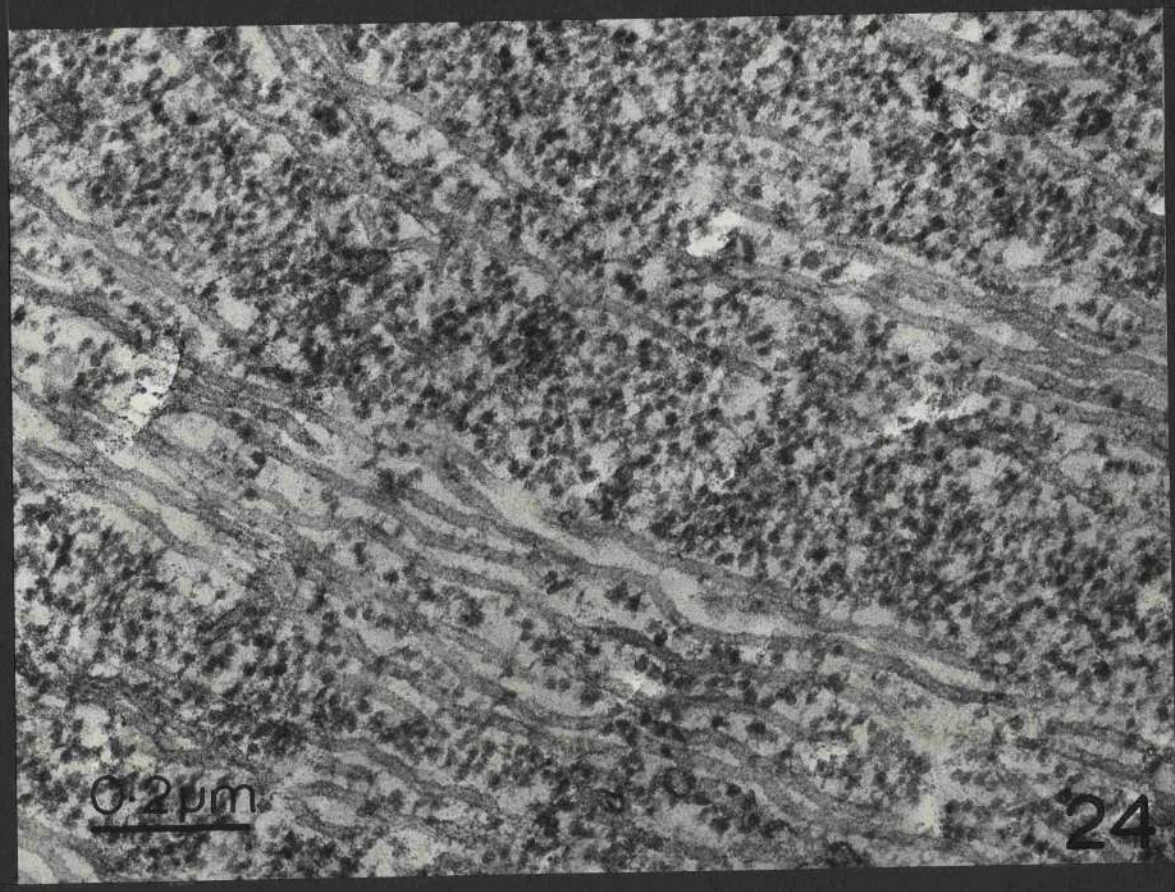

0·2 µm
24

Fig. 25.

Transverse section through part of a nutritive tube. In transverse section the microtubules show as dark staining circles 20nm in diameter with a lighter centre. There is a clear cytoplasmic region around most microtubules (arrowed).

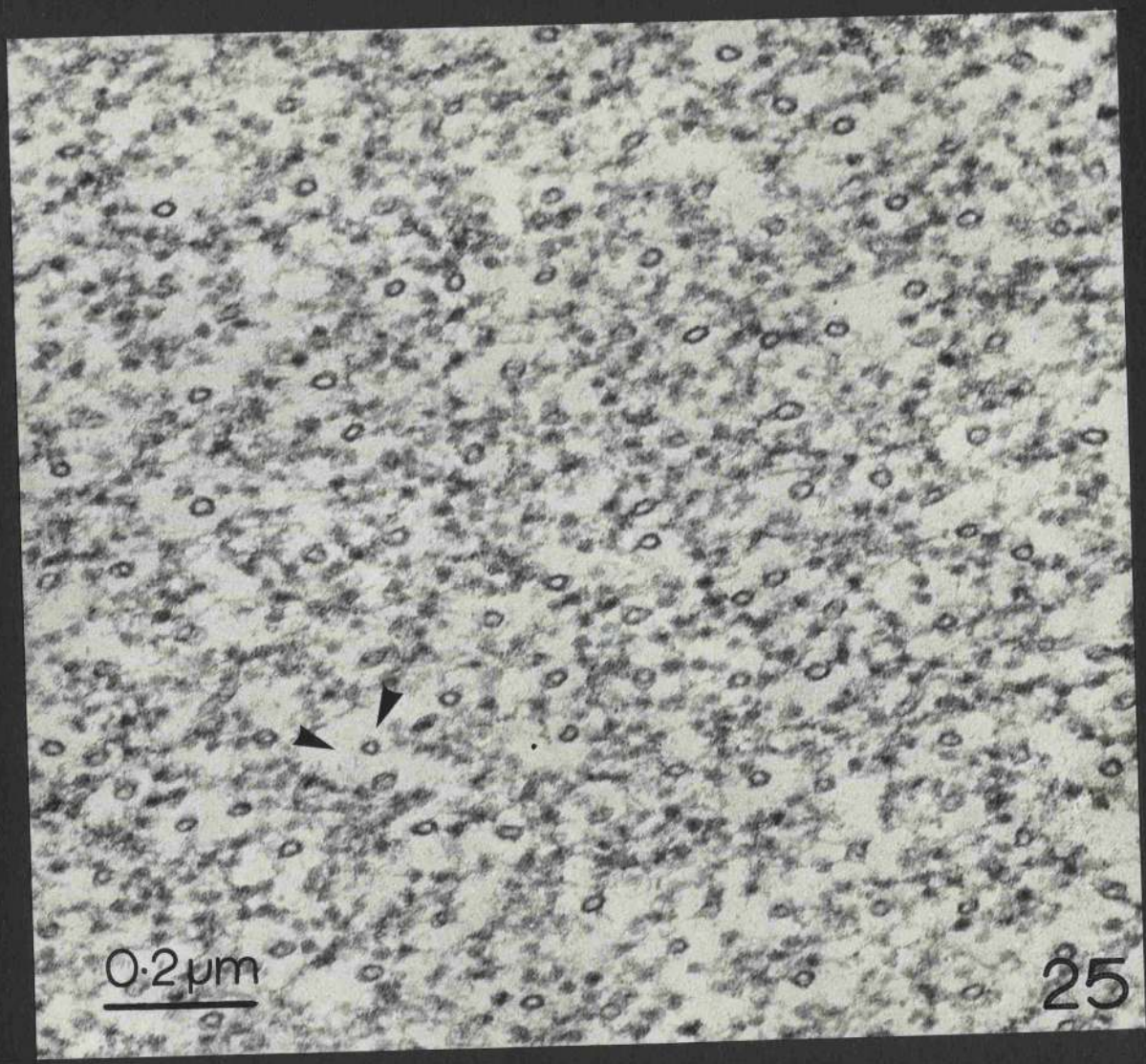
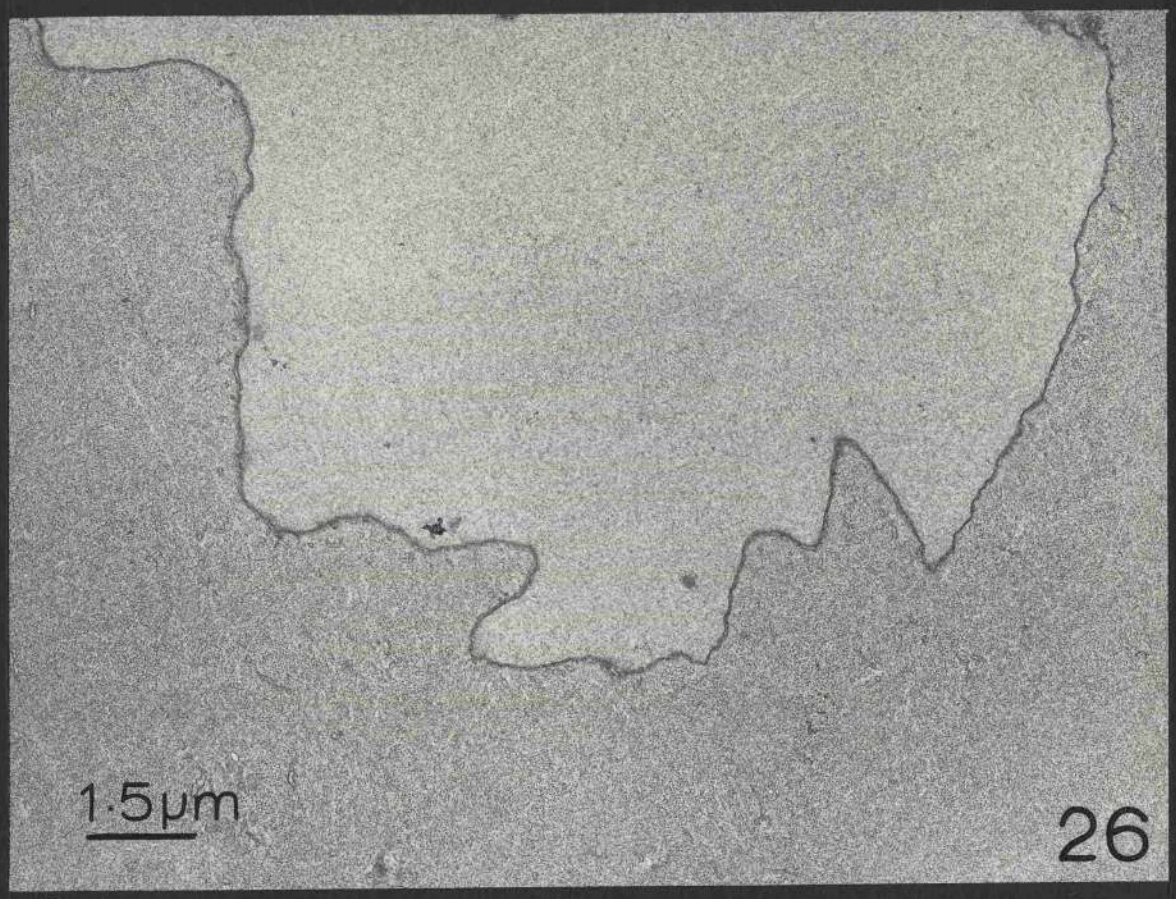

0·2 µm
25

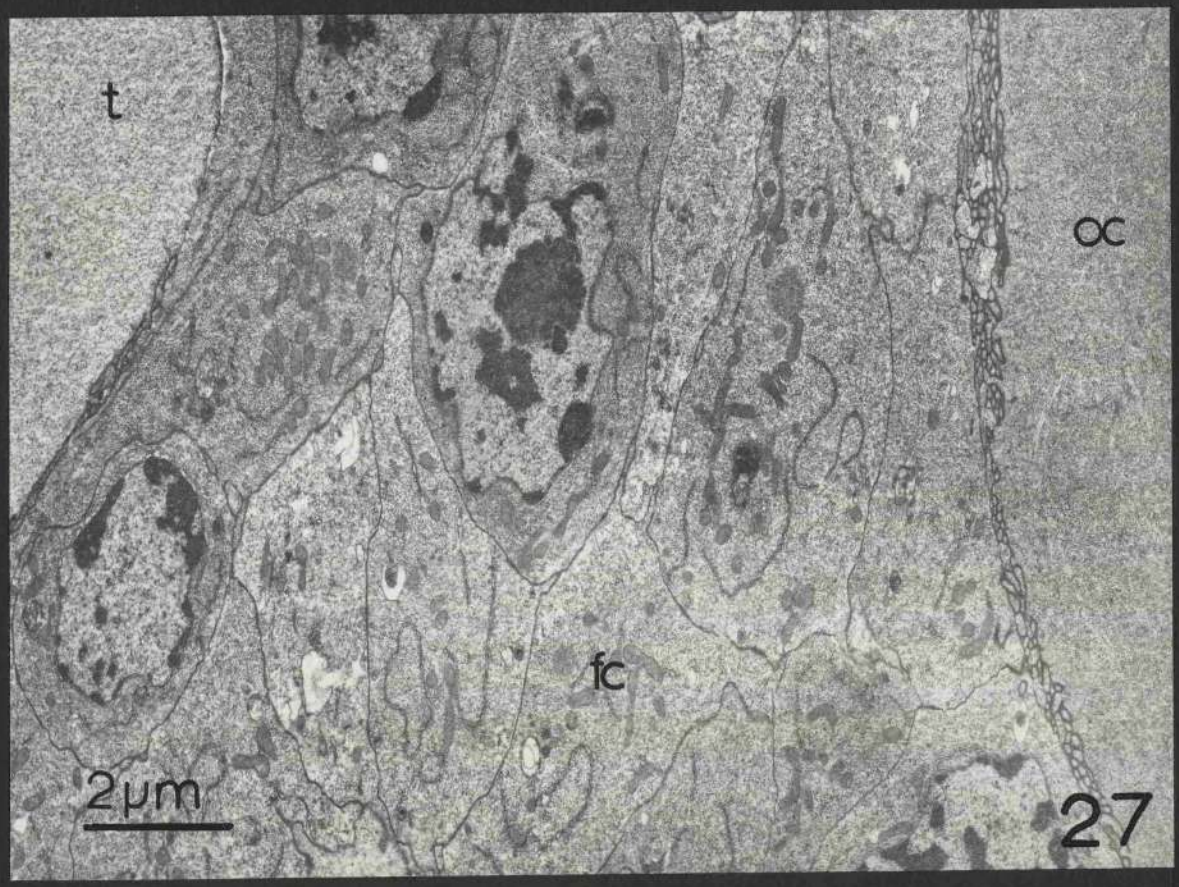
Fig. 26.

The oocyte cytoplasm is packed with ribosomes. The oocyte nucleus is also shown in this micrograph and no nucleoli are evident.

Fig. 27.

Low power electron micrograph showing the interdigitating border between the follicle cells (fc) and the cytoplasm of the oocyte (oc). On the left is part of a nutritive tube (t).


1·5µm
26


t
oc
fc
2µm
27

Figs. 28 - 32 are micrographs of ovarioles taken with a Carl Zeiss photomicroscope fitted with crossed polaroids.

Fig. 28.

The terminal filament (f), the trophic core (c) and the nutritive tubes are strongly birefringent.

Fig. 29.

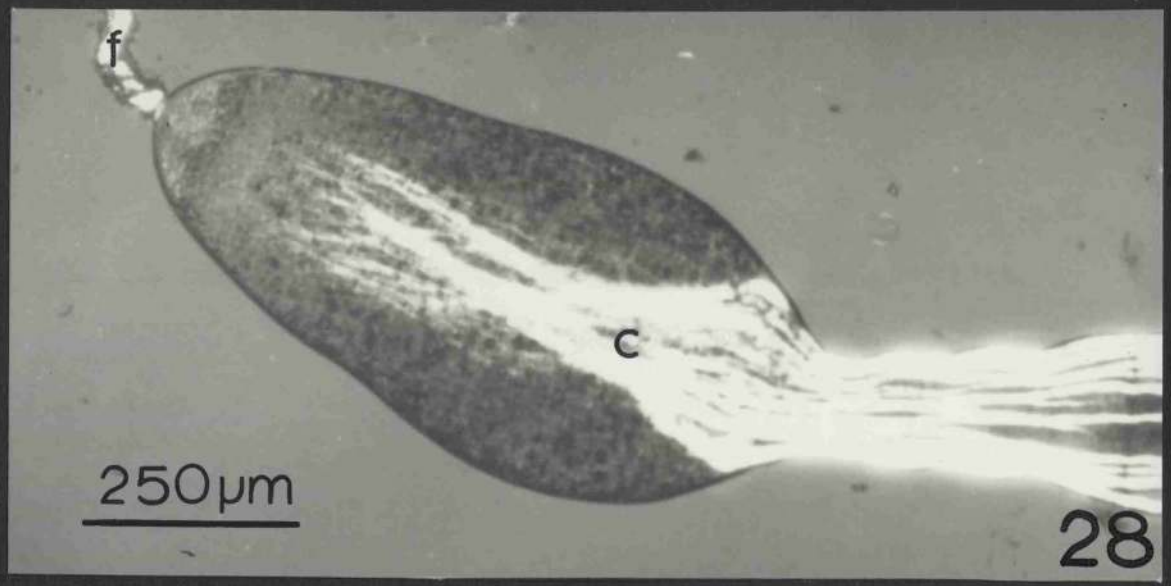
Faint birefringent extensions can be seen running outwards from the trophic core.

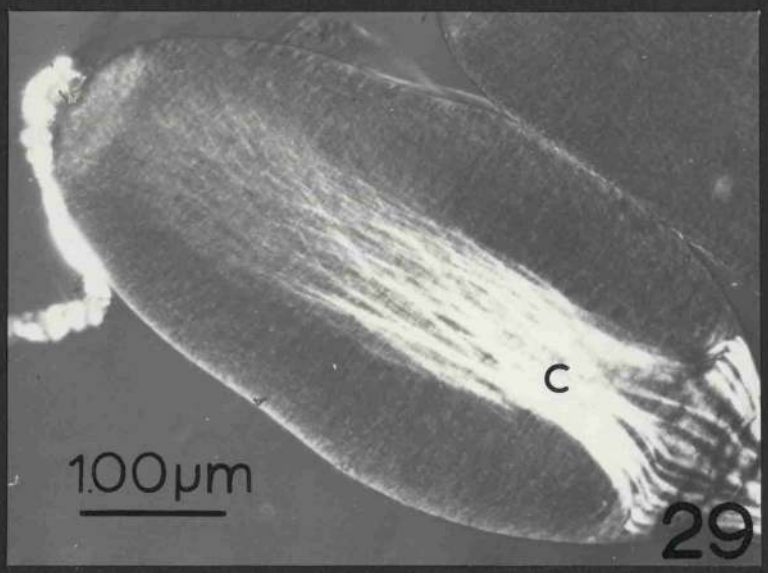
Fig. 30.

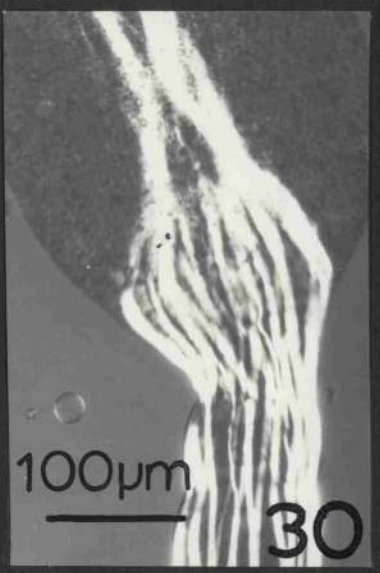
Nutritive tubes pass back from the core in a basket fashion around the prefollicular region.

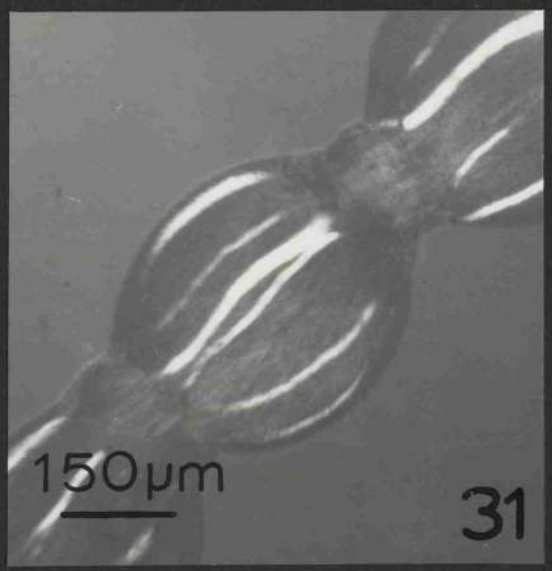
Figs. 31 and 32.

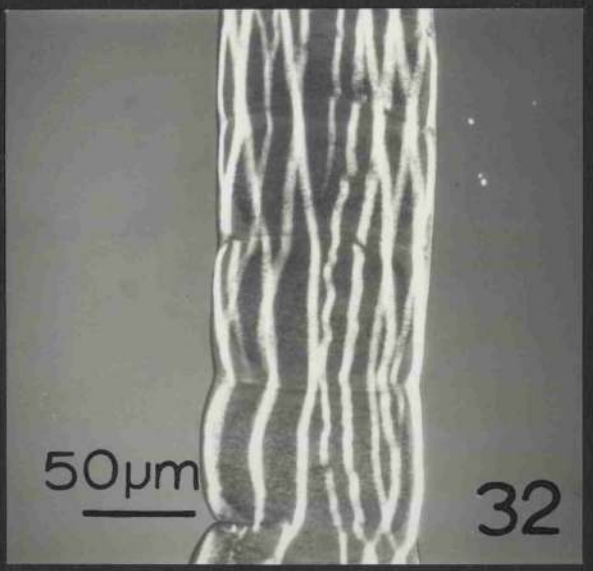
Tubes pass back around the follicles to each oocyte in the ovariole.


f
c
250µm
28


c
100µm
29


100µm
30


150µm
31


50µm
32

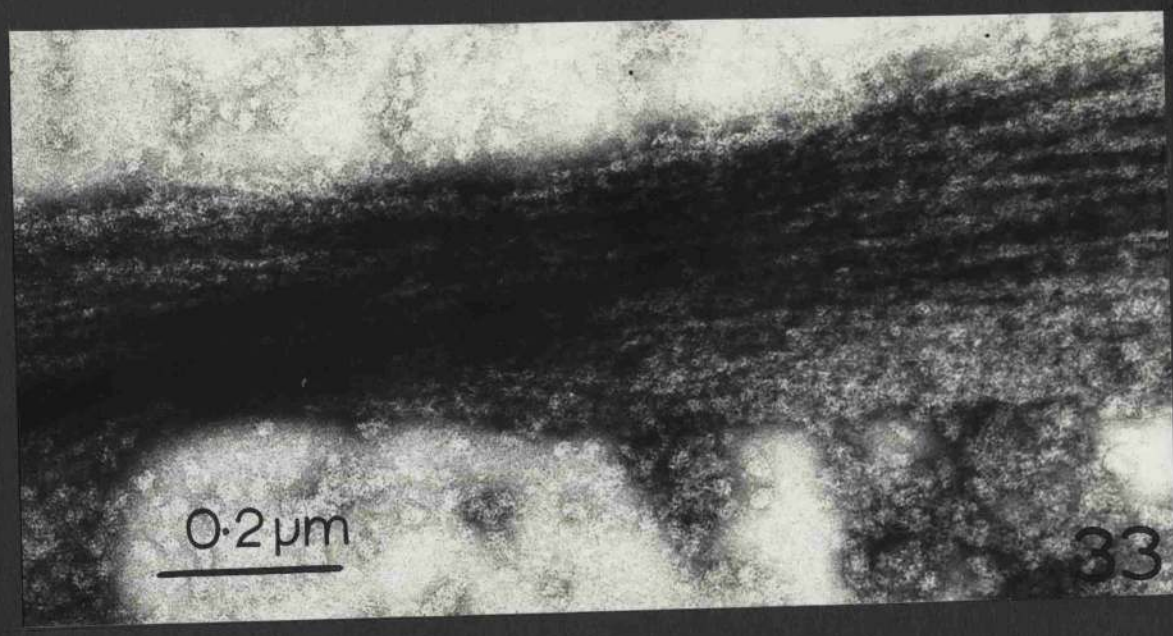
Figs. 33 and 34 are electron micrographs of microtubules isolated from the nutritive tubes of ovarioles in dilute solutions of formaldehyde and negatively stained with uranyl acetate and phosphotungstic acid.

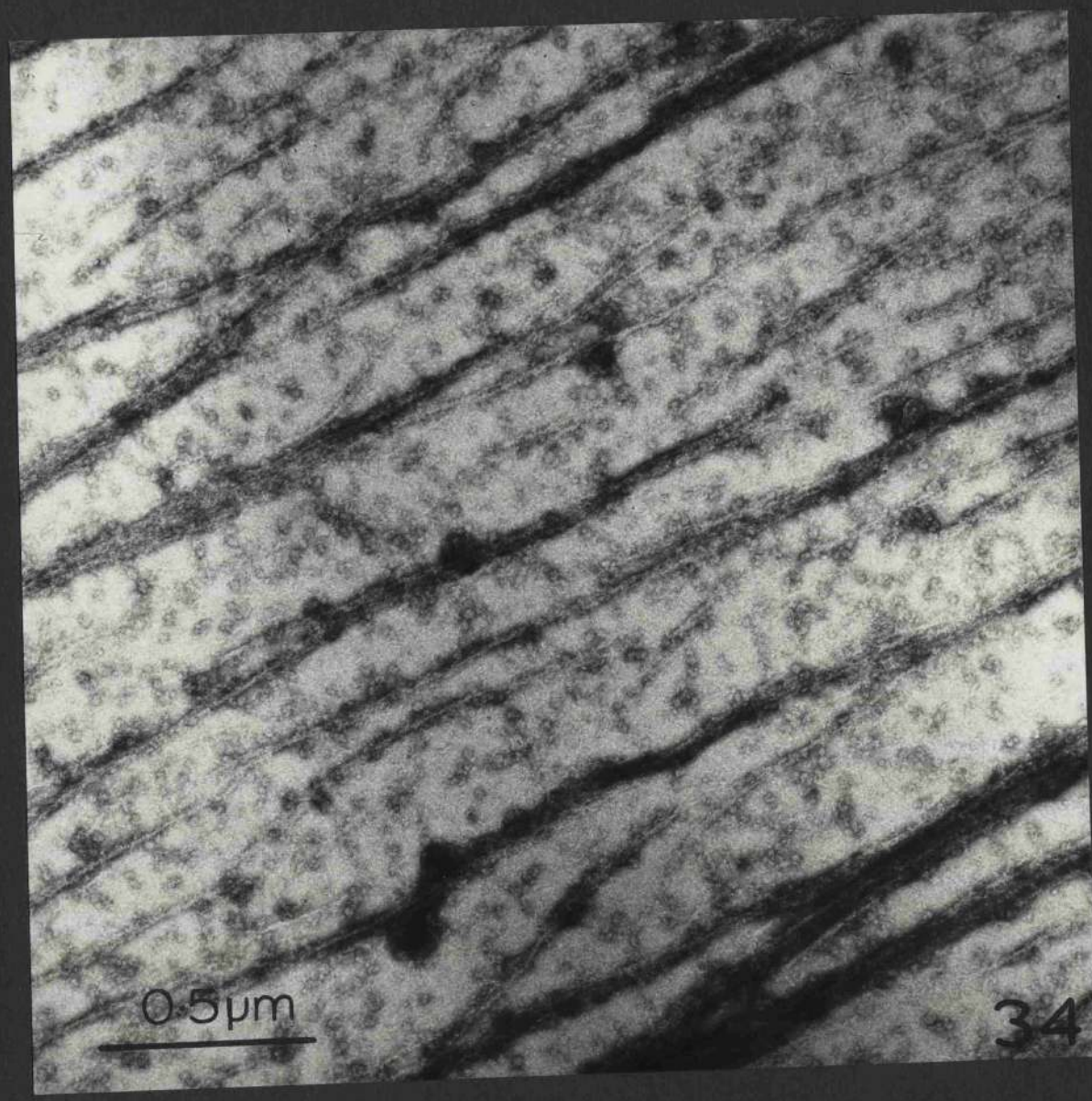
Fig. 33.

Aggregations of microtubules are bounded by clusters of ribosomes.

Fig. 34.

Where the microtubules have scattered and spread onto the grids they are seen in roughly parallel rows and are surrounded by and even 'encrusted' with ribosomes. The substructure of the microtubules is not well preserved with this isolation procedure.


0·2 μm
33


0·5 μm
34

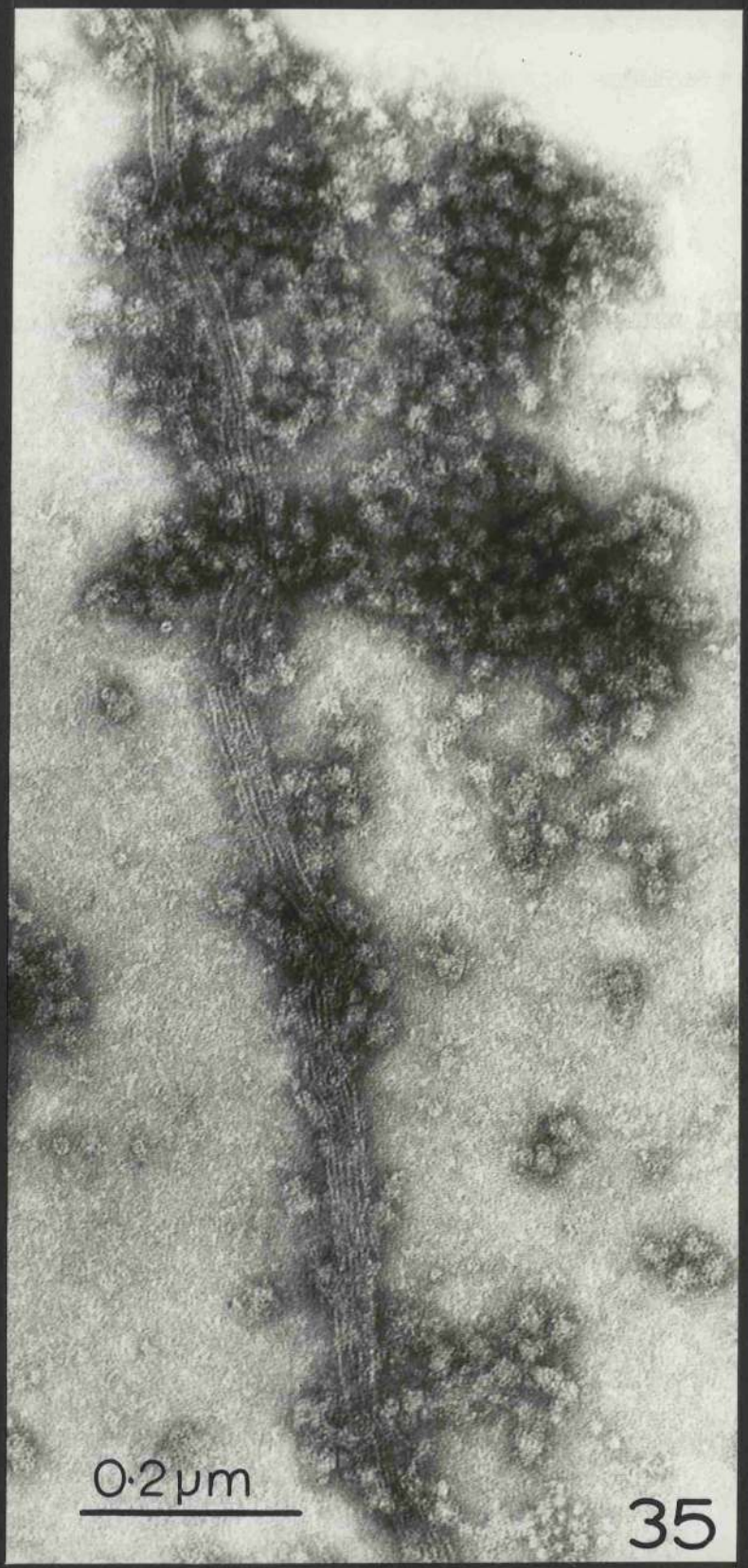
Figs. 35 and 36 are electron micrographs of microtubules which have been isolated from ovarioles in buffered hexylene glycol and negatively stained.

Fig. 35.

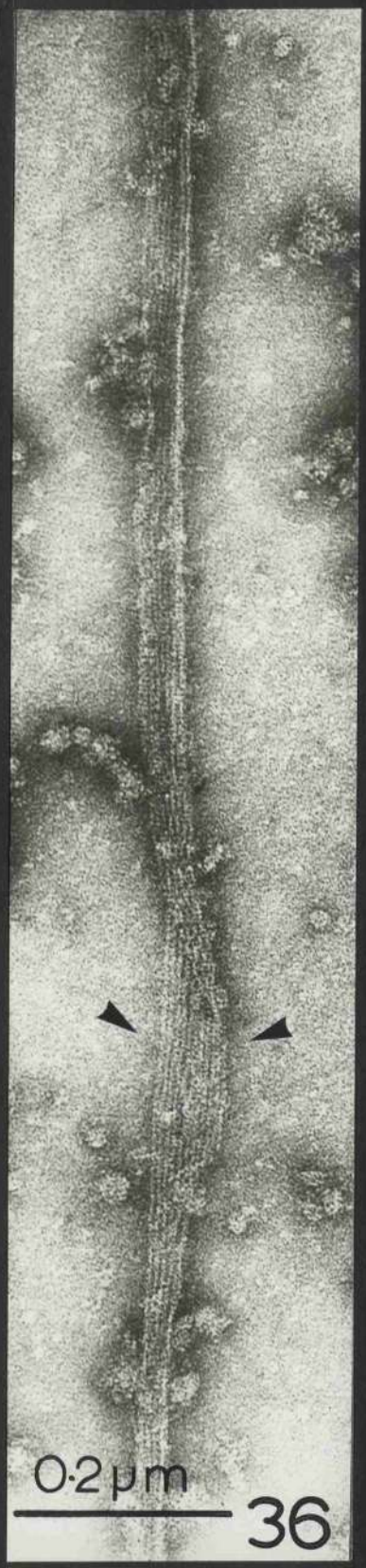
Where individual microtubules are free from ribosomal clusters they collapse onto the grids revealing the sub-filamentous substructure within their walls. A periodicity of approximately 4 nm can also be seen along each sub-filament.

Fig. 36.

In some places individual microtubules have split open and where this has occurred (arrowed) the number of sub-filaments within the walls of a single tubule can be counted accurately.



35



36

Figs. 37 - 41 show freeze-etched fractures of microtubules within nutritive tubes.

Fig. 37.

Longitudinally fractured microtubules in nutritive tubes. With this technique the microtubules appear straight in comparison to the 'wavy' image observed in thin sections.

Fig. 38.

Part of a transversely fractured nutritive tube showing transversely fractured microtubules. A 'clear zone' can be seen around individual microtubules and this etches to the same extent as the 'cores' of the microtubules.


0·2 µm
37

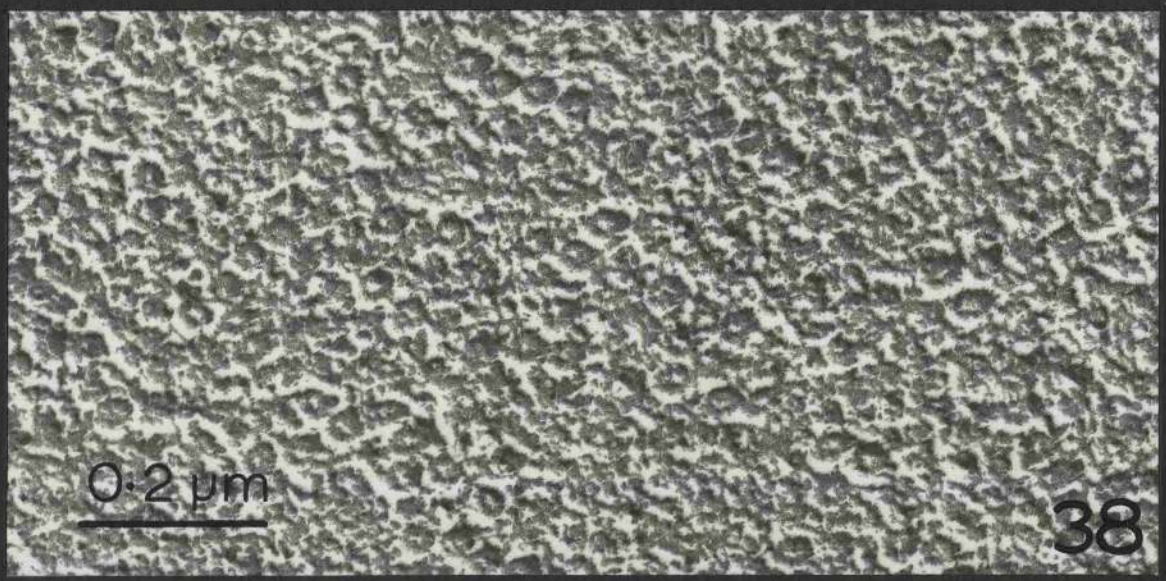

0·2 µm
38

Fig. 39.

Longitudinally fractured microtubules in a frozen-etched nutritive tube. The microtubules fractured both at their surfaces (arrowed) and internally, revealing their tubular structure. The outer surfaces of the microtubules disply longitudinal striations which correspond with sub-filaments.

Fig. 40.

Obliquely fractured microtubules in a frozen-etched nutritive tube. The arrangement of sub-filaments in the wall of the microtubules are particularly clear in this case (arrowed).

Fig. 41.

Longitudinally fractured microtubules in a frozen-etched nutritive tube. Apparent cross-bridges between closely adjacent microtubules are commonly found. This effect might be a technical artifact.

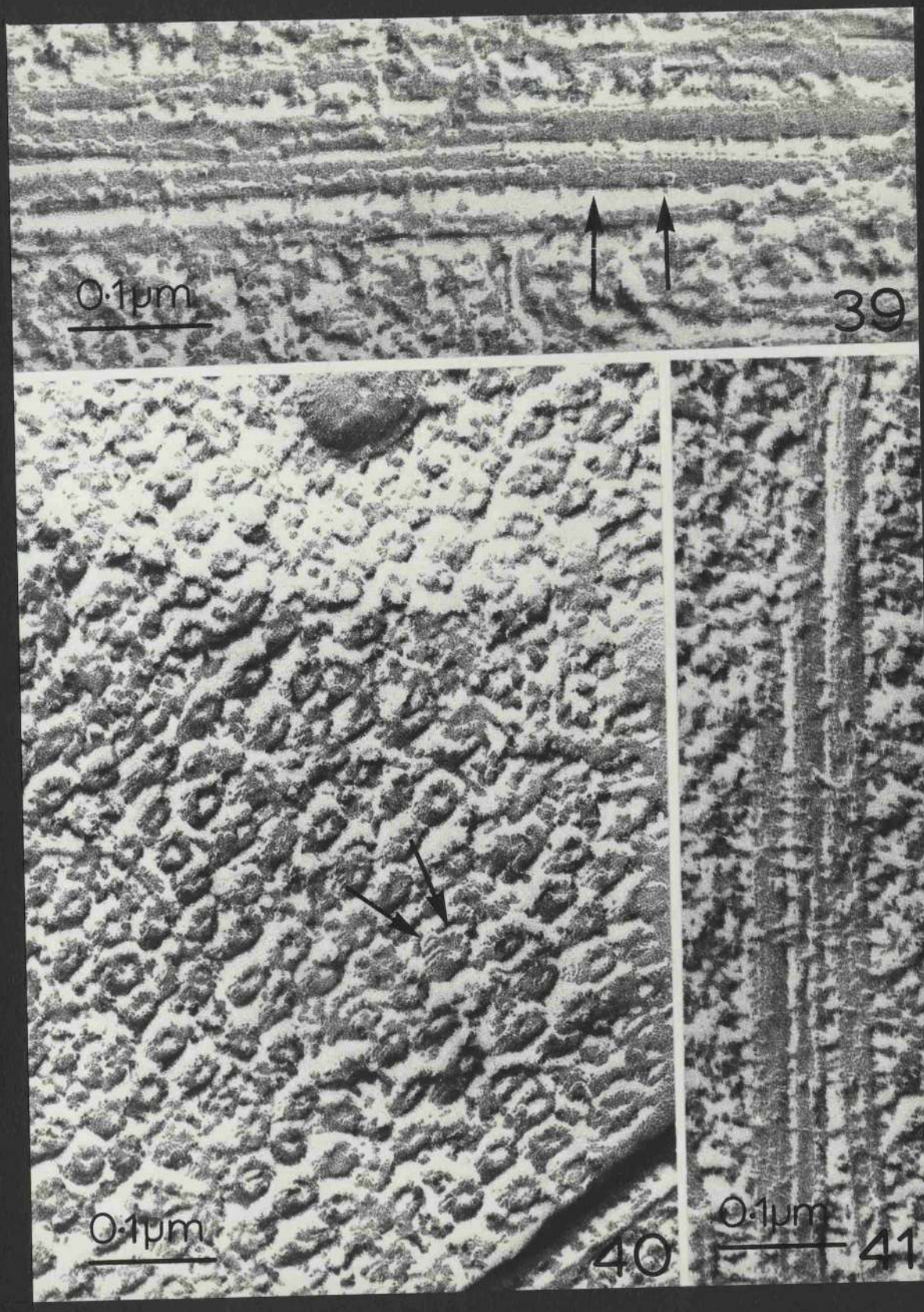
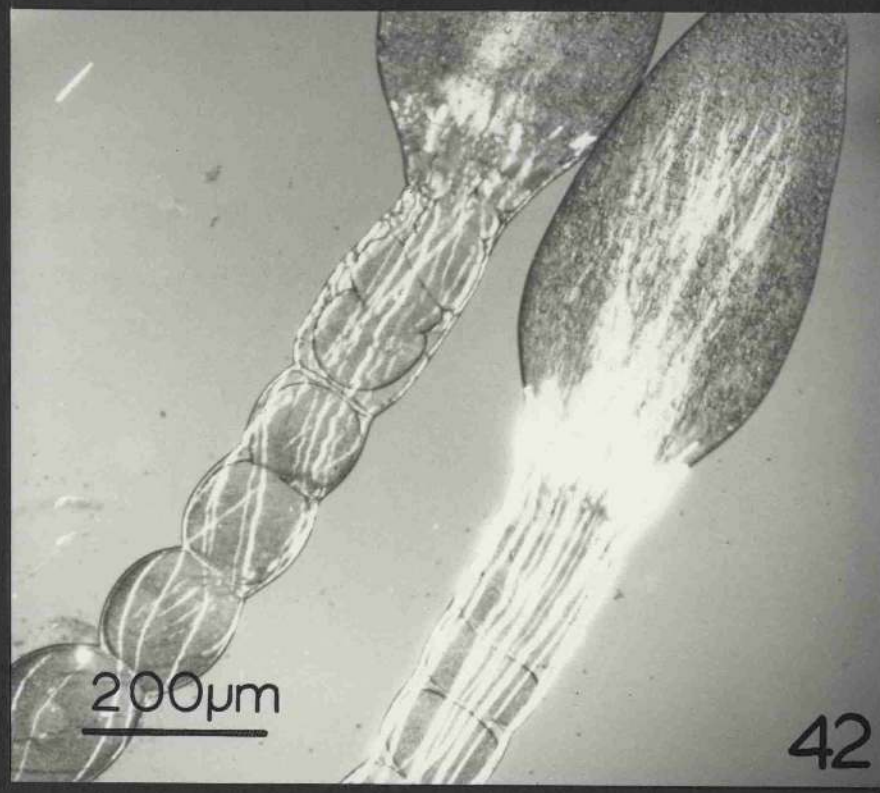

0·1μm
39
0·1μm
40
0·1μm
41

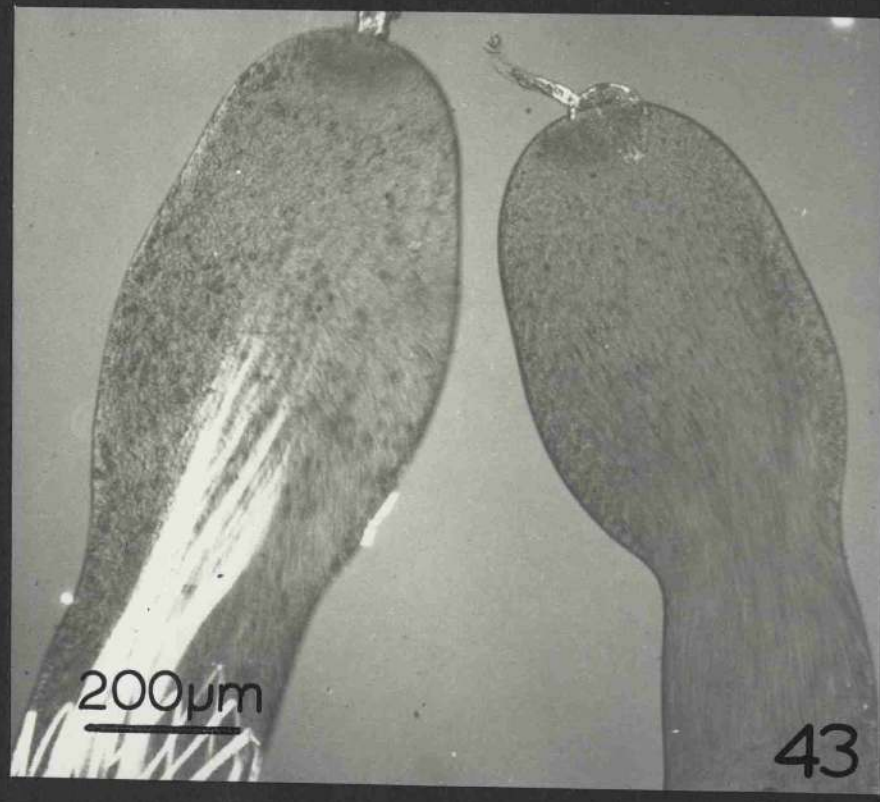
Fig. 42.

The cold treated ovariole on the left shows greatly reduced birefringence in comparison with the control ovariole on the right.

Fig. 43.

The colchicine treated ovariole on the right has lost its birefringence and trophic nuclei have flowed back into the neck of the ovariole. This movement produces the streaming effect in this long exposure micrograph. A normal ovariole is shown on the left.


200µm
42


200µm
43

Figs. 44 - 46 are electron micrographs of transverse sections of parts of parts of nutritive tubes after treatment with different concentrations of colchicine for 3h.

Fig. 44.

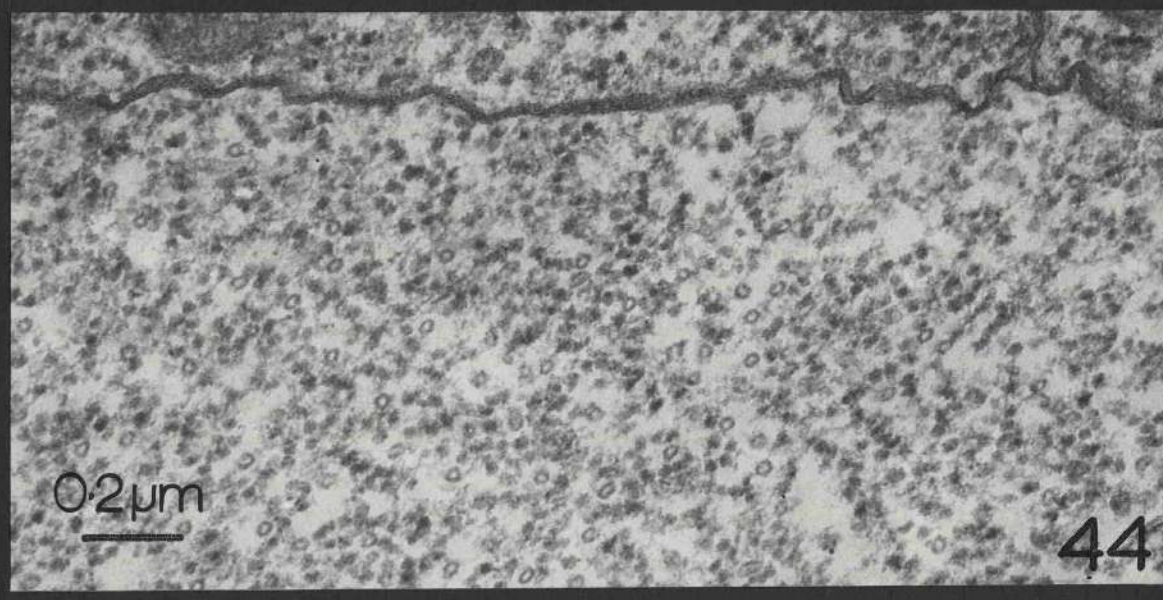
Nutritive tubes treated with 0.1% colchicine are indistinguishable from untreated tubes.

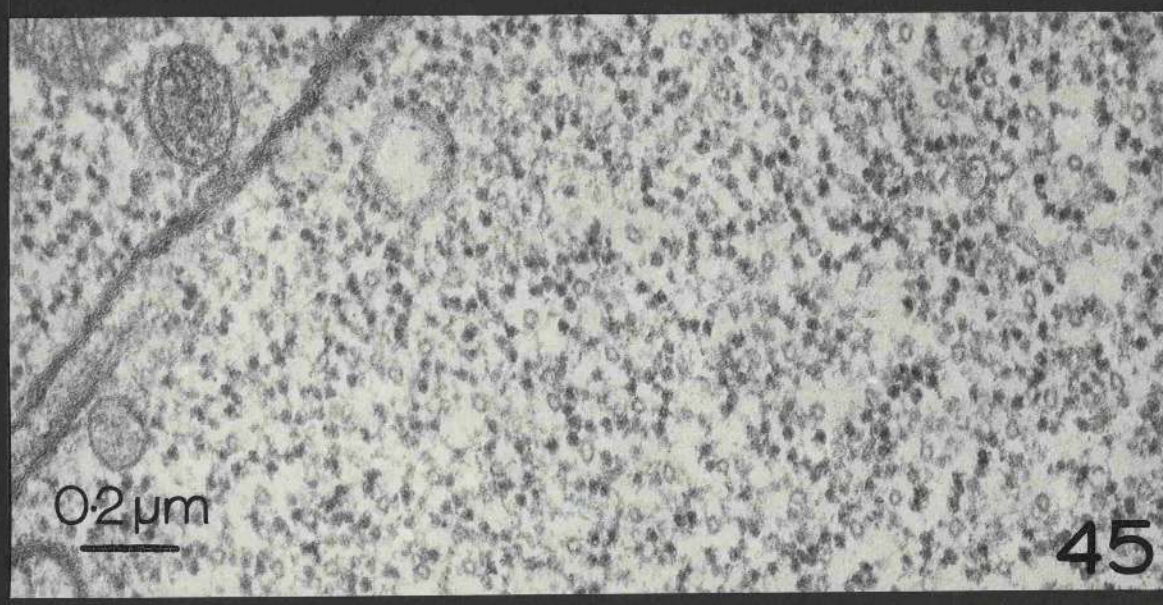
Fig. 45.

After treatment with 1.0% colchicine the number of microtubules within the nutritive tubes is markedly reduced.

Fig. 46.

Microtubules are rare (arrowed) in the nutritive tubes of ovarioles treated with 2.0% colchicine and the ribosomes spread evenly over transverse sections of the tubes.


0·2μm
44


0·2μm
45

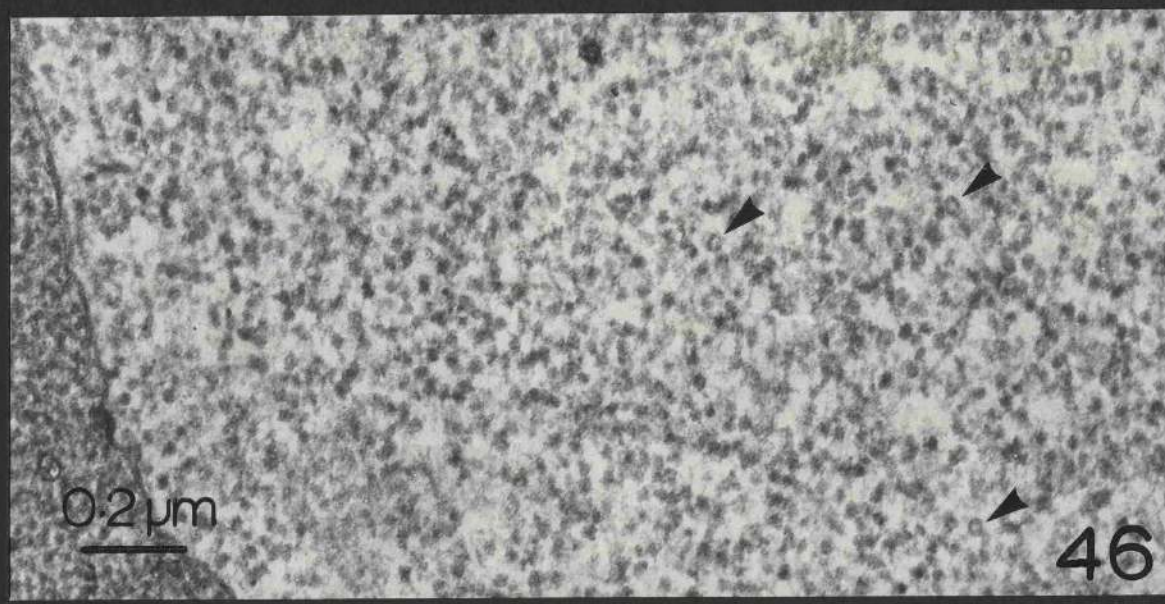

0·2μm
46

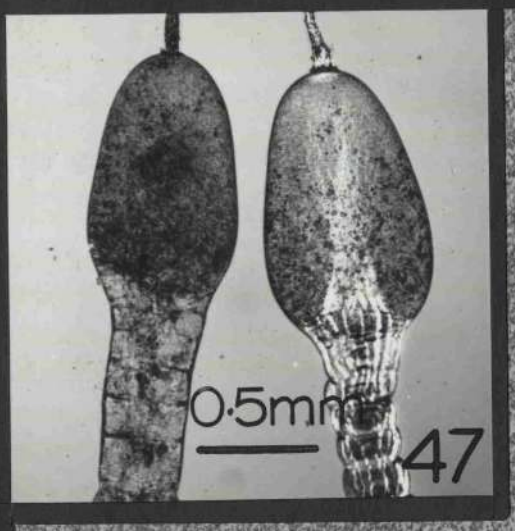
Fig. 47.

Photomicrograph in polarised light of the anterior region of two ovarioles. The ovariole on the left has been bathed for 6h in 10^{-3} M vinblastine (VBL). The one on the right was kept in Ringer.

Figs. 48 - 53 are electron micrographs of ovarioles after treatment for 6h with 10^{-3} M VBL.

Fig. 48.

Transverse section through part of an ovariole showing an oocyte (oo), and a nutritive tube (nt) surrounded by follicle cells. Crystals (cr) can be seen in the tube and the follicle cells. In the tube the crystals are cut in transverse section, while in the follicle cells the crystals are randomly oriented.


0·5mm
47

Cr
nt
Cr
Cr
Cr
Cr
1 µm
oc
48

Fig. 49.

Transverse section through a nutritive tube. Crystal structures (cr) can be seen, also in transverse section. The VBL has destroyed the majority of the microtubules leaving arcs and circles of ribosomes.

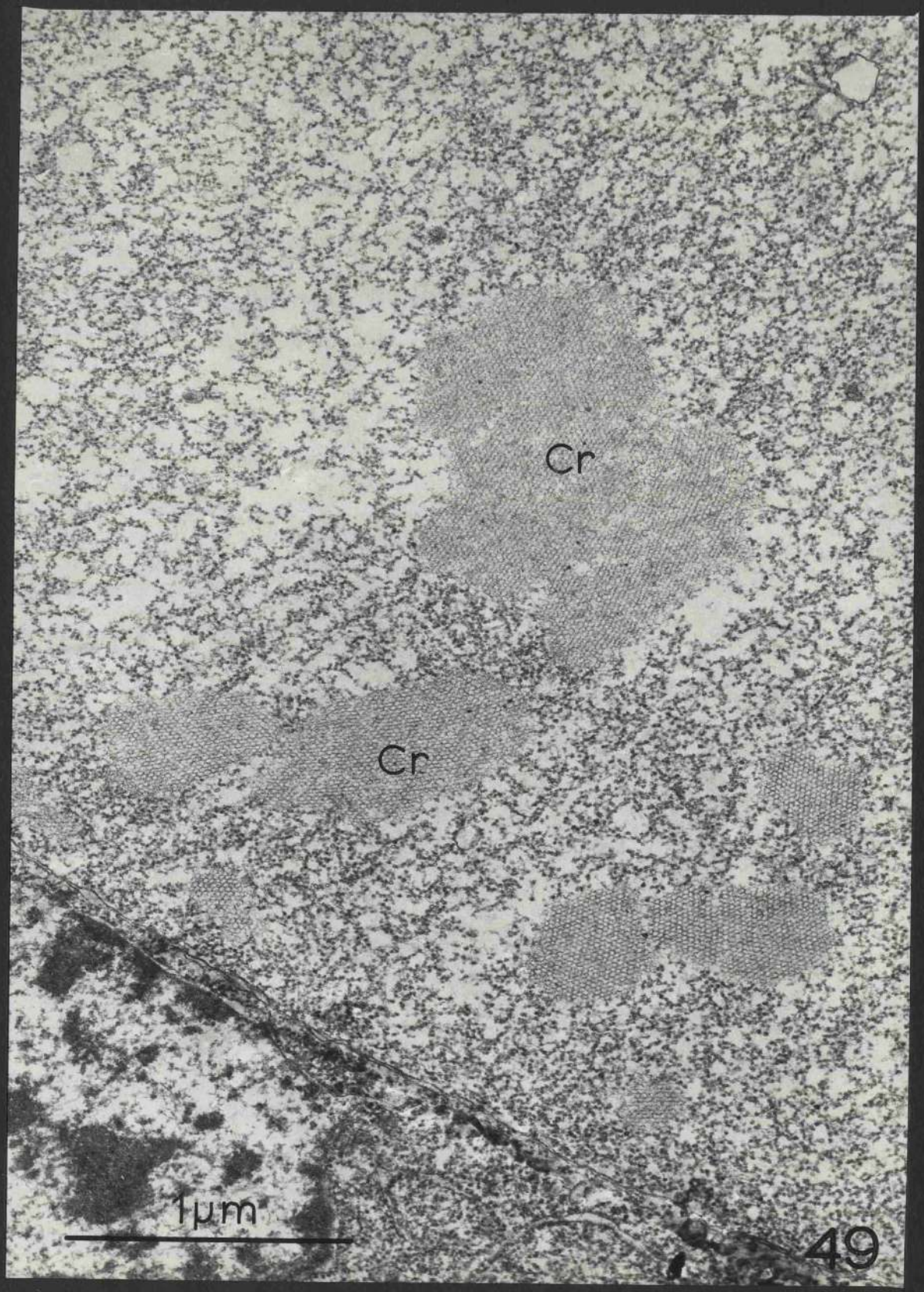
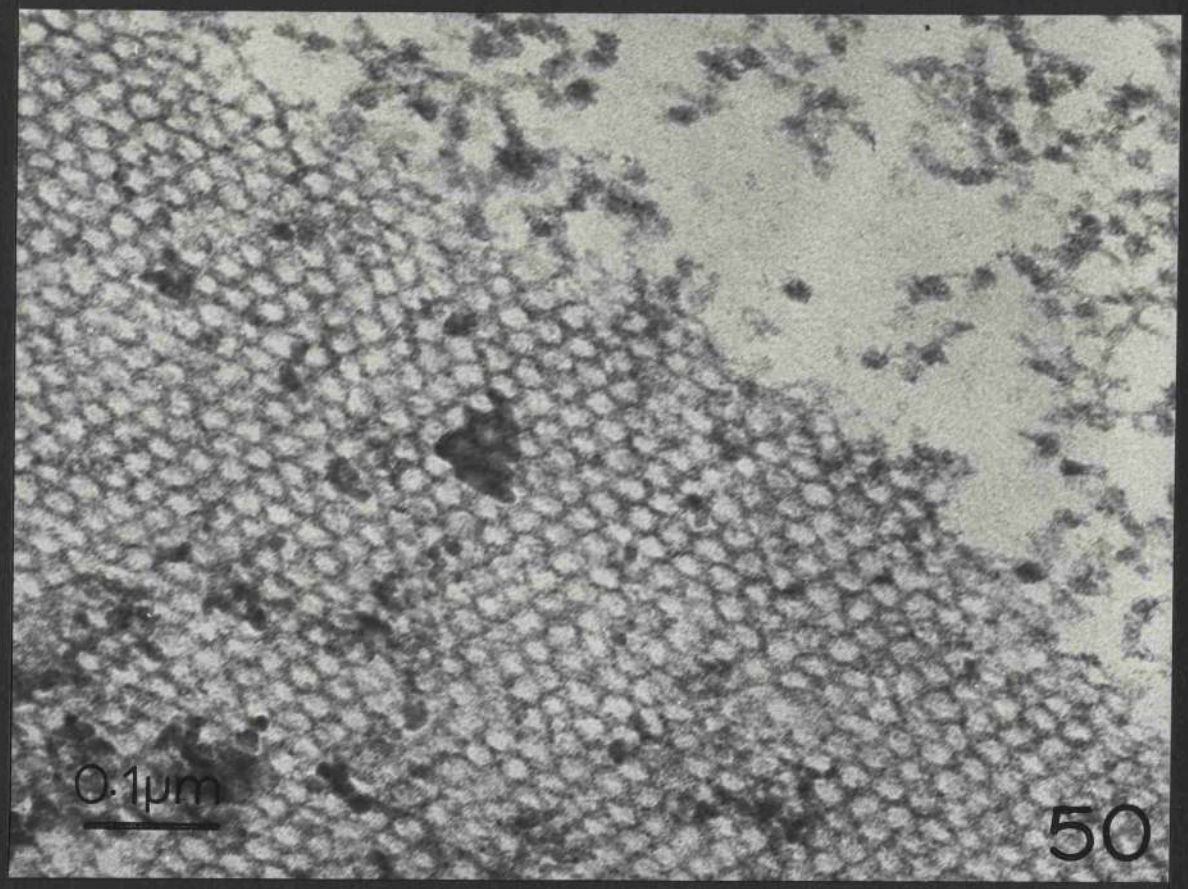

Cr
Cr
1µm
49

Fig. 50.

Transverse section through a crystal, showing its regular hexagonal sub-units.

Fig. 51.

Longitudinal section of a nutritive tube, showing the co-existance of microtubules and crystals. In such sections of nutritive tubes the crystals and the microtubules, when present, are always seen in longitudinal section.


0·1μm
50

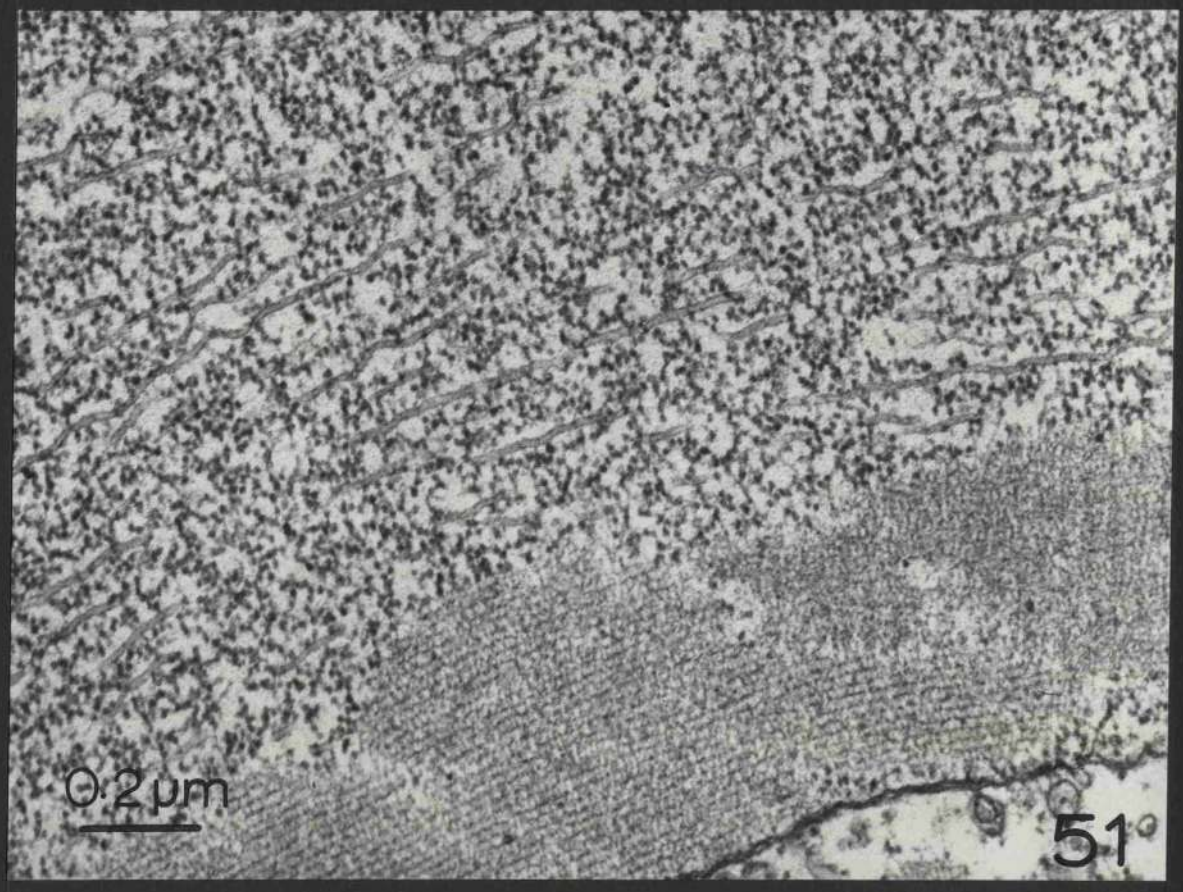
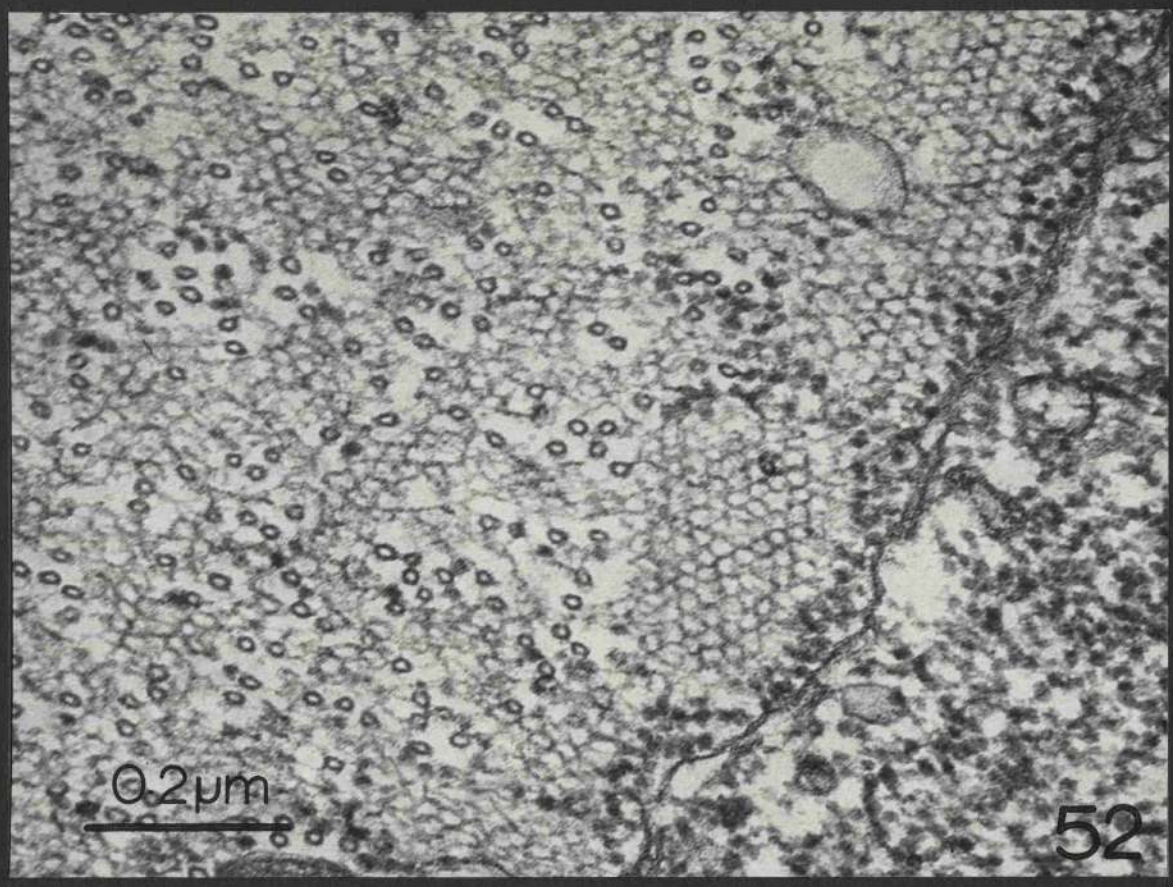

0·2μm
51

Fig. 52.

Transverse section through part of a nutritive tube showing microtubules surrounded by an extensive crystal lattice. There are few contacts between microtubules and crystals.

Fig. 53.

Section through the cytoplasm of a nutritive cell. Rows of ribosomes appear to be in continuity with the crystal, which is seen in longitudinal section. There is an out-of-step arrangement of ribosomes in adjacent rows.


0.2μm
52

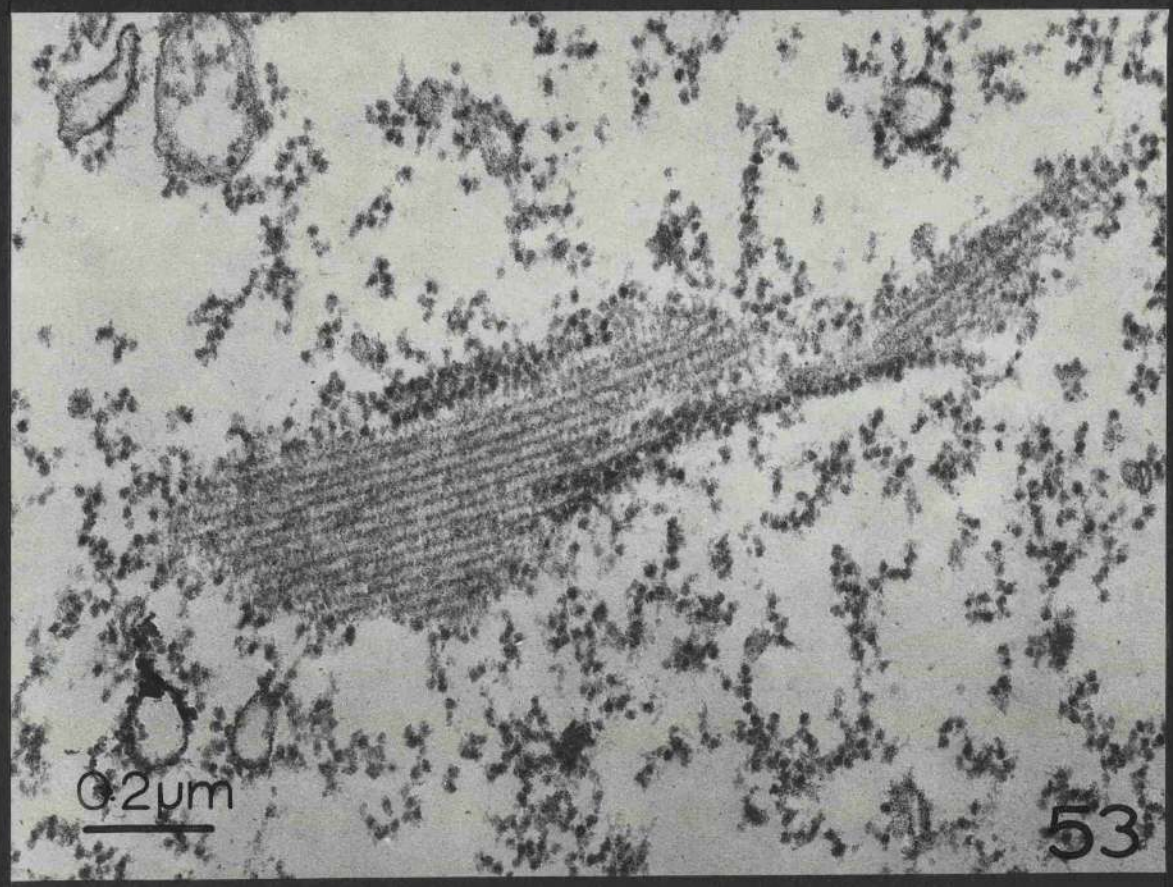

0.2μm
53

Fig. 54.

Electron micrograph of a transverse section through part of a nutritive tube within an ovariole after treatment with 2×10^{-3} M VBL for 6h. Crystal structures spread over much of the transverse section of the nutritive tube. In places the crystal structure is loose and web-like (arrowed), which contracts with the regular hexagonal sub-units of compact crystals. In this tube, there is an almost total absence of free ribosomes.


0.5μm
54

Fig. 55.

Electron micrograph of a transverse section of part of a nutritive tube within an ovariole which had been treated for 3h with 10^{-4} M VBL. Crystals (cr) can be seen within the nutritive tube and also in the cytoplasm of one of the surrounding follicle cells. With this concentration of VBL, microtubules are visible in those parts of the nutritive tube not occupied by crystals.

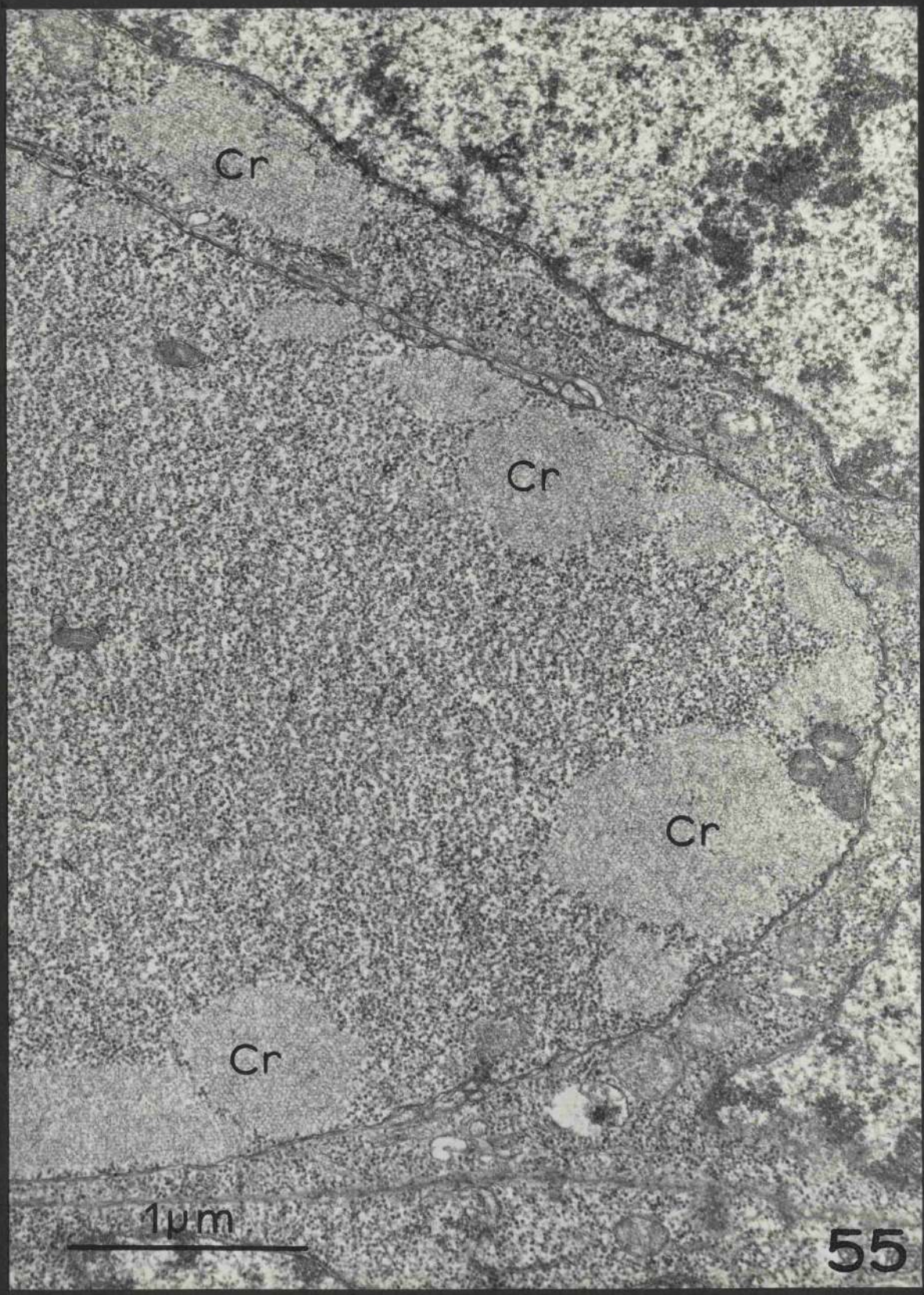

Cr
Cr
Cr
Cr
1µm
55

Fig. 56.

Section of the precipitate formed when vinblastine was added to supernatants of homogenates of ovaries of Notonecta. In some regions circular profiles are discernible on close examination (see arrows).

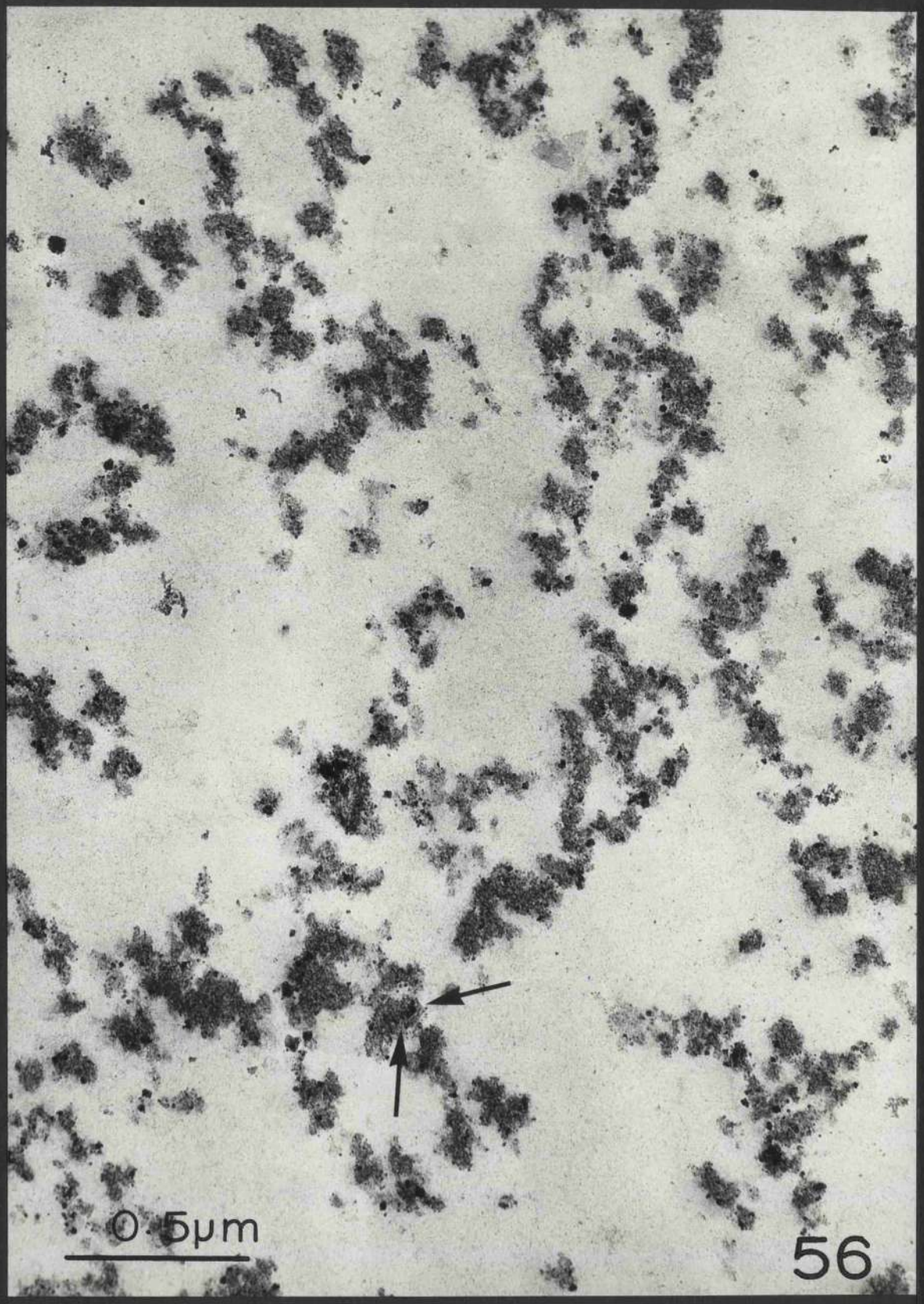

0.5μm
56

Fig. 57.

Scans of acrylamide gels. Proteins separated by electrophoresis and stained with fast green.

A) Represents proteins in 110,000 g supernatant of homogenates of whole ovaries of Notonecta.

B) Proteins from flagella of Chlamydomonas.

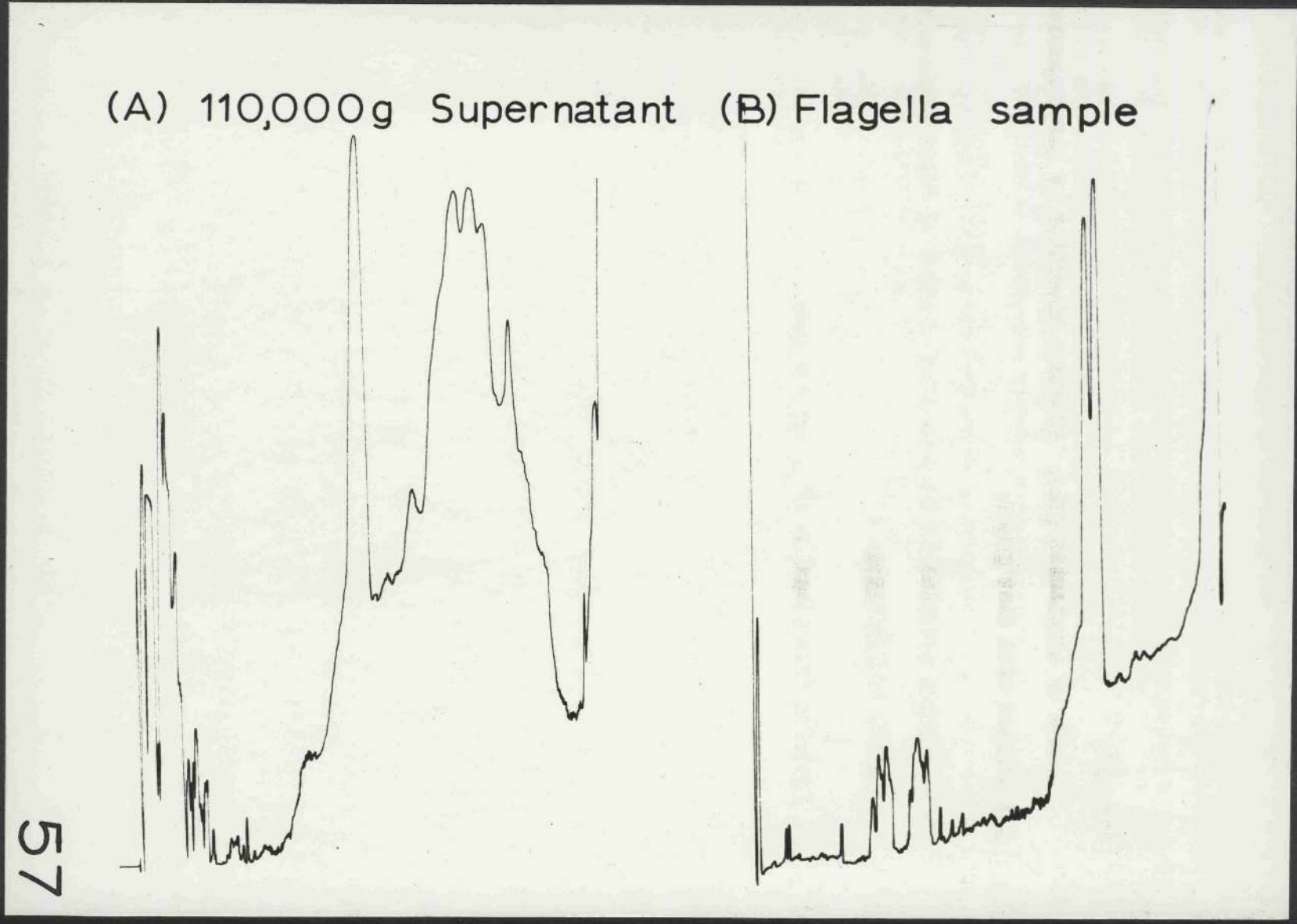

(A) 110,000g Supernatant
(B) Flagella sample

Fig. 58.

Scans of acrylamide gels. Proteins separated by electrophoresis and stained with fast green.

C) Proteins remaining in supernatant after 110,000 g supernatants of ovaries have been treated with VBL.

D) Proteins in VBL precipitate.

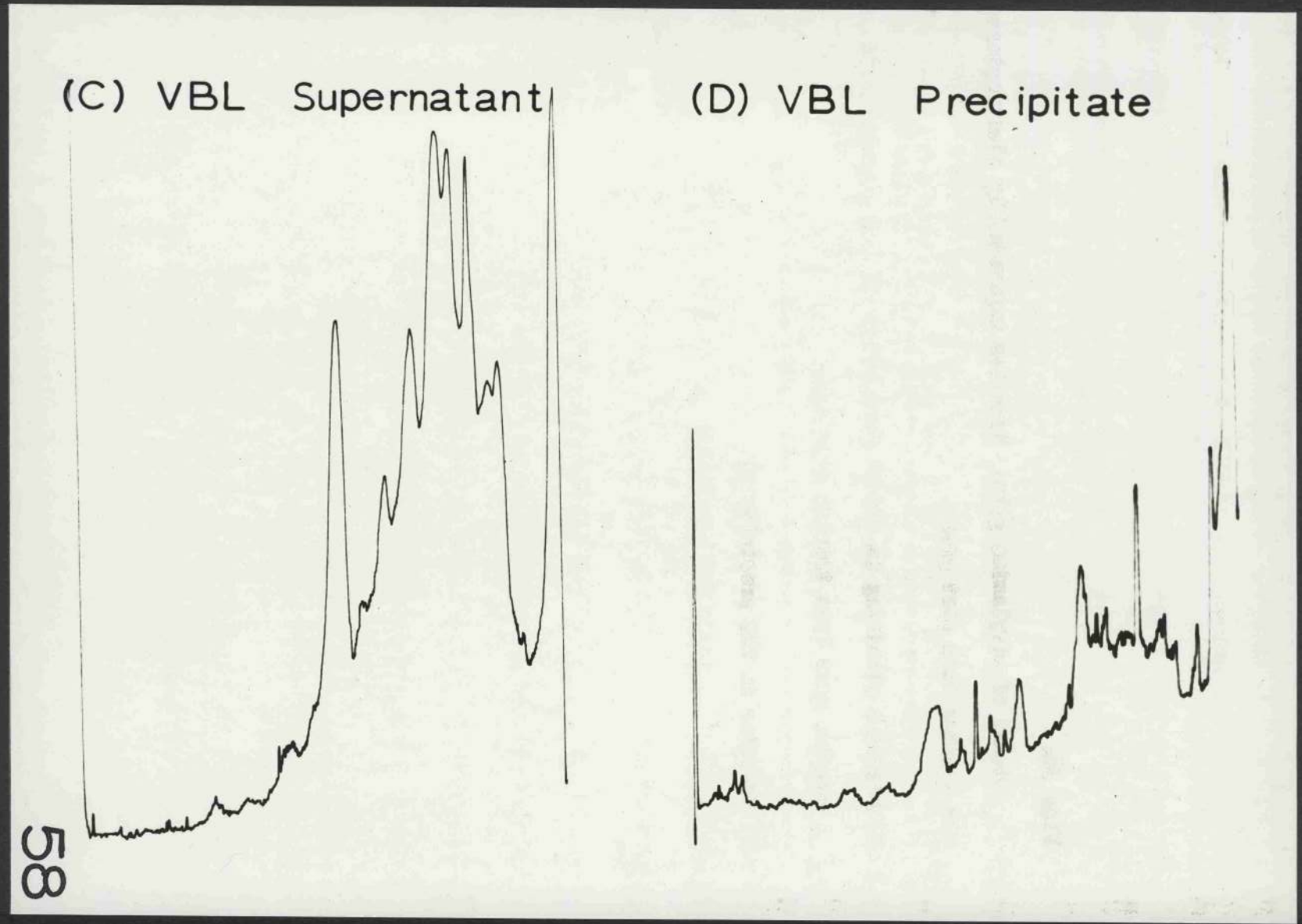
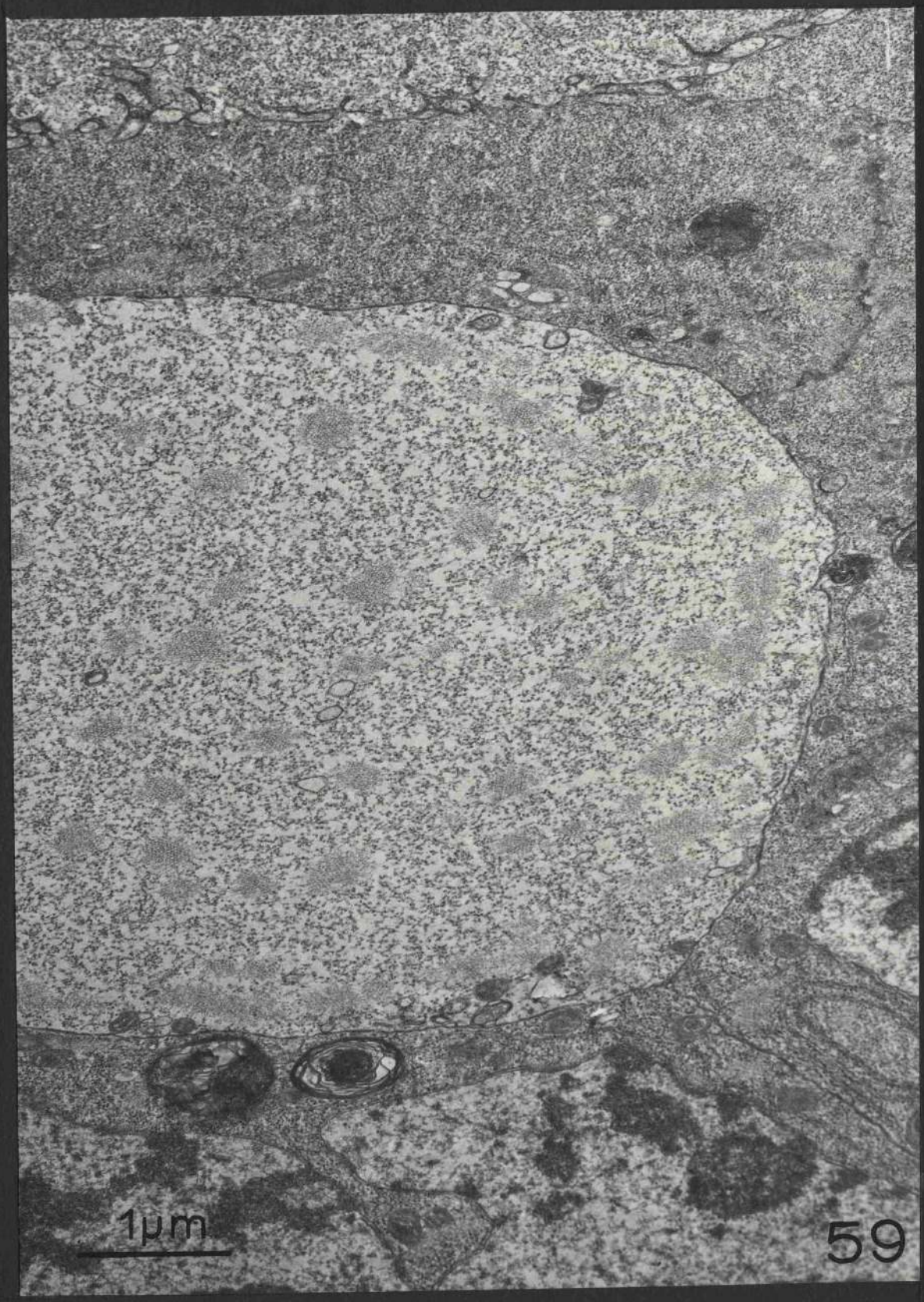

(C) VBL Supernatant
(D) VBL Precipitate

Fig. 59.

Electron micrograph of a transverse section through part of a nutritive tube from an ovariole which had been pre-treated for 3h with 0.1% colchicine, followed by 10^{-3} M VBL for 3h. Crystals composed of regularly shaped sub-units can be seen amongst the microtubules within the nutritive tube.


1µm
59

Figs. 60 and 61 are electron micrographs of ovarioles after treatment with 1.0% colchicine for 3h, followed by treatment with 10^{-3} M VBL for a further 3h.

Fig. 60.

After pretreatment with 1.0% colchicine, VBL induces the formation of feathery aggregates within the nutritive tubes.

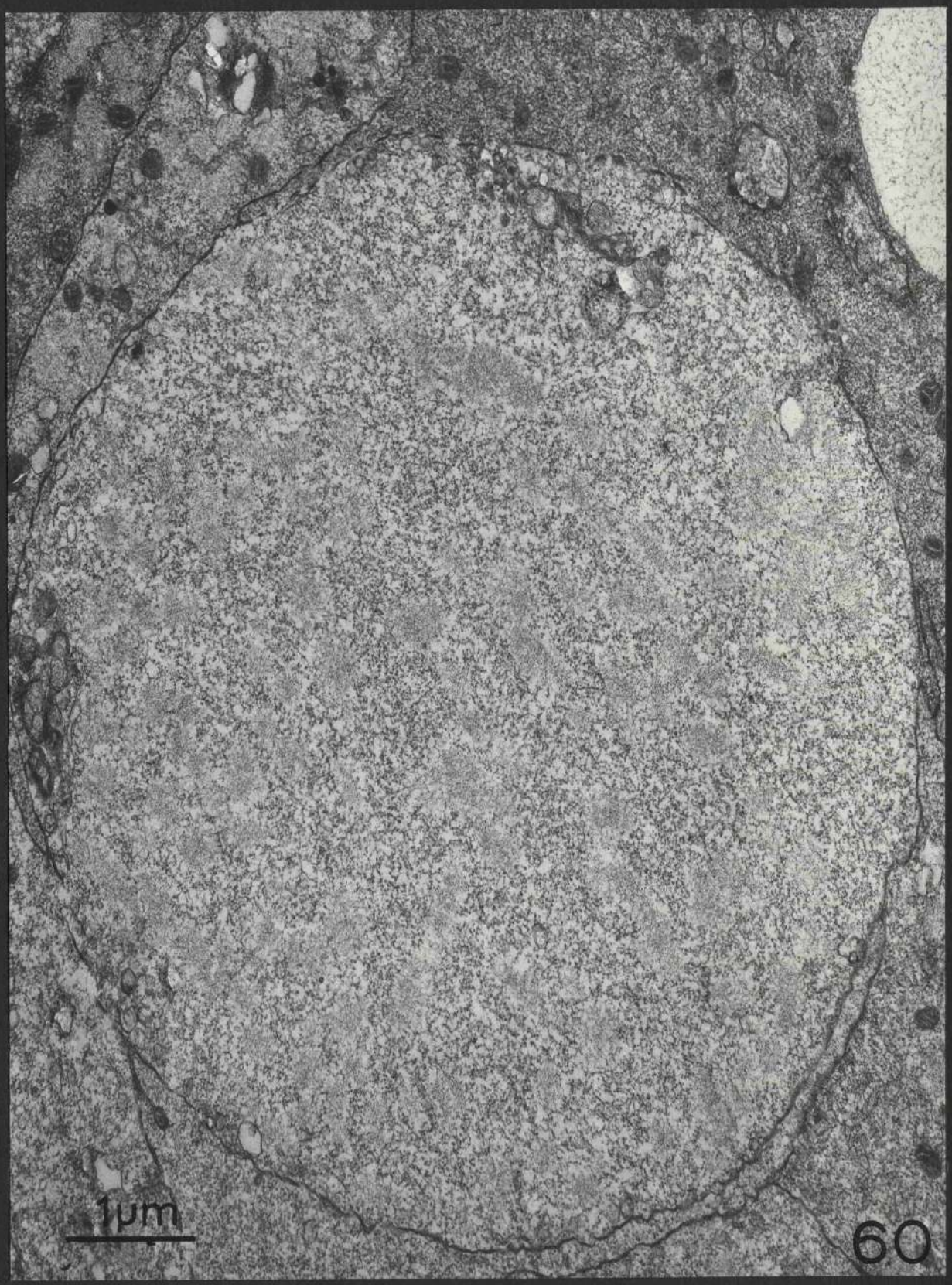

1µm
60

Fig. 61.

In contrast with the crystals composed of regular sub-units which form in nutritive tubes after treatment with VBL, feathery aggregates are induced by VBL in nutritive tubes which had been pretreated with 1.0% colchicine.

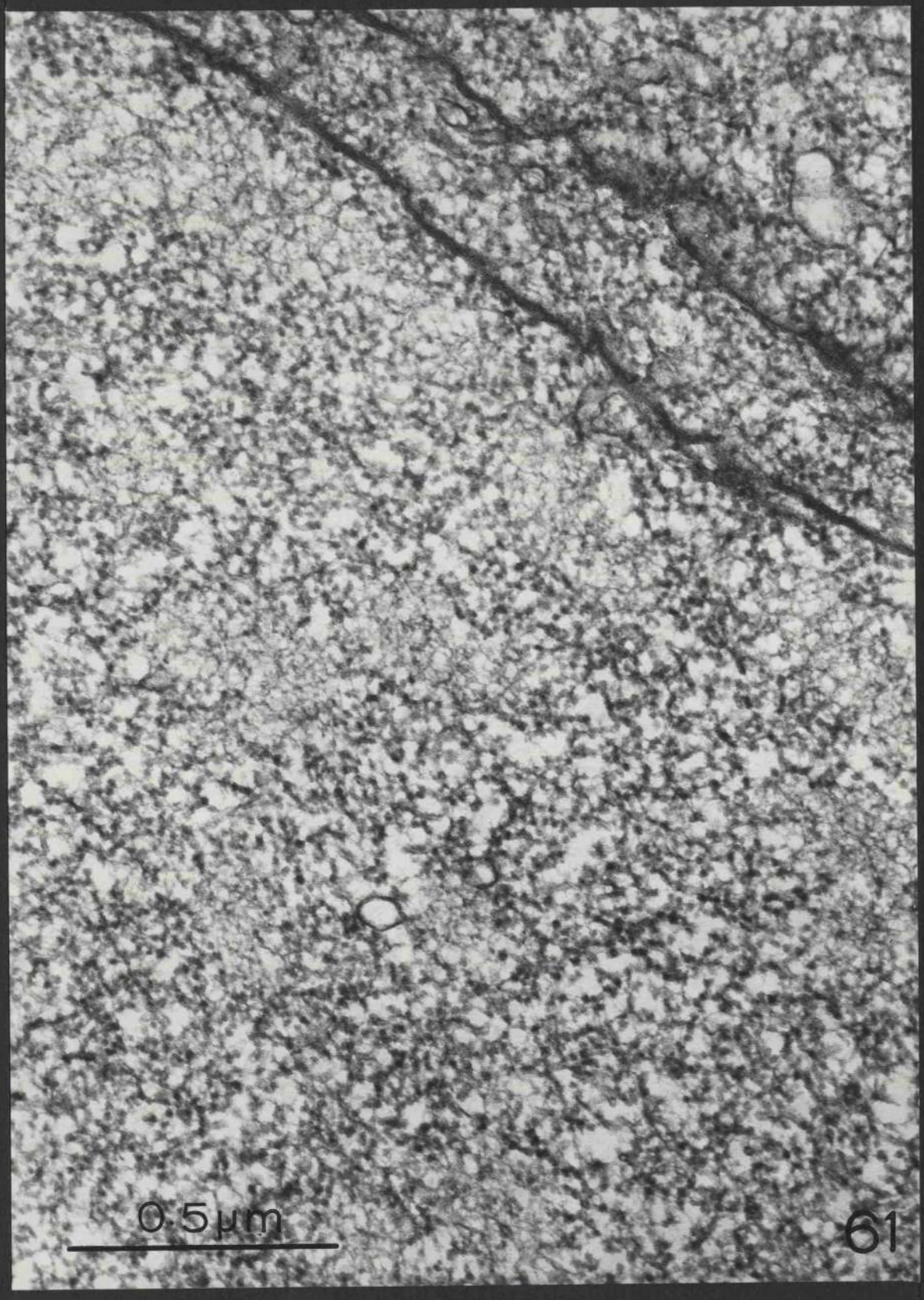
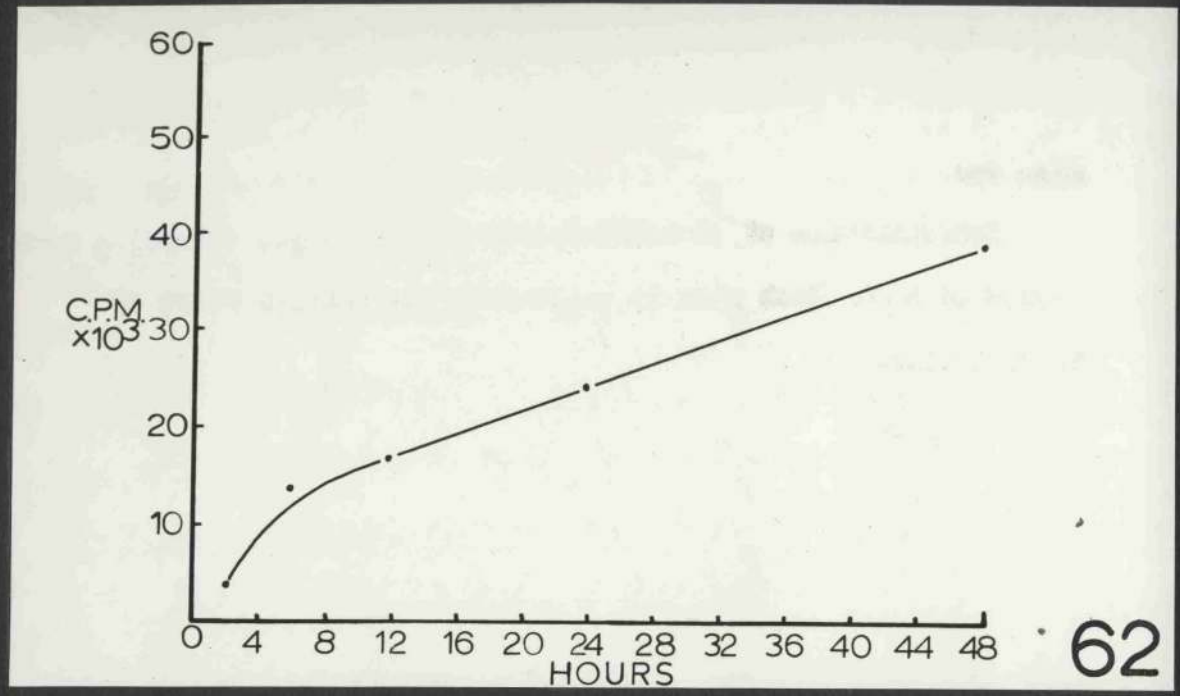

0.5 µm
61

Fig. 62.

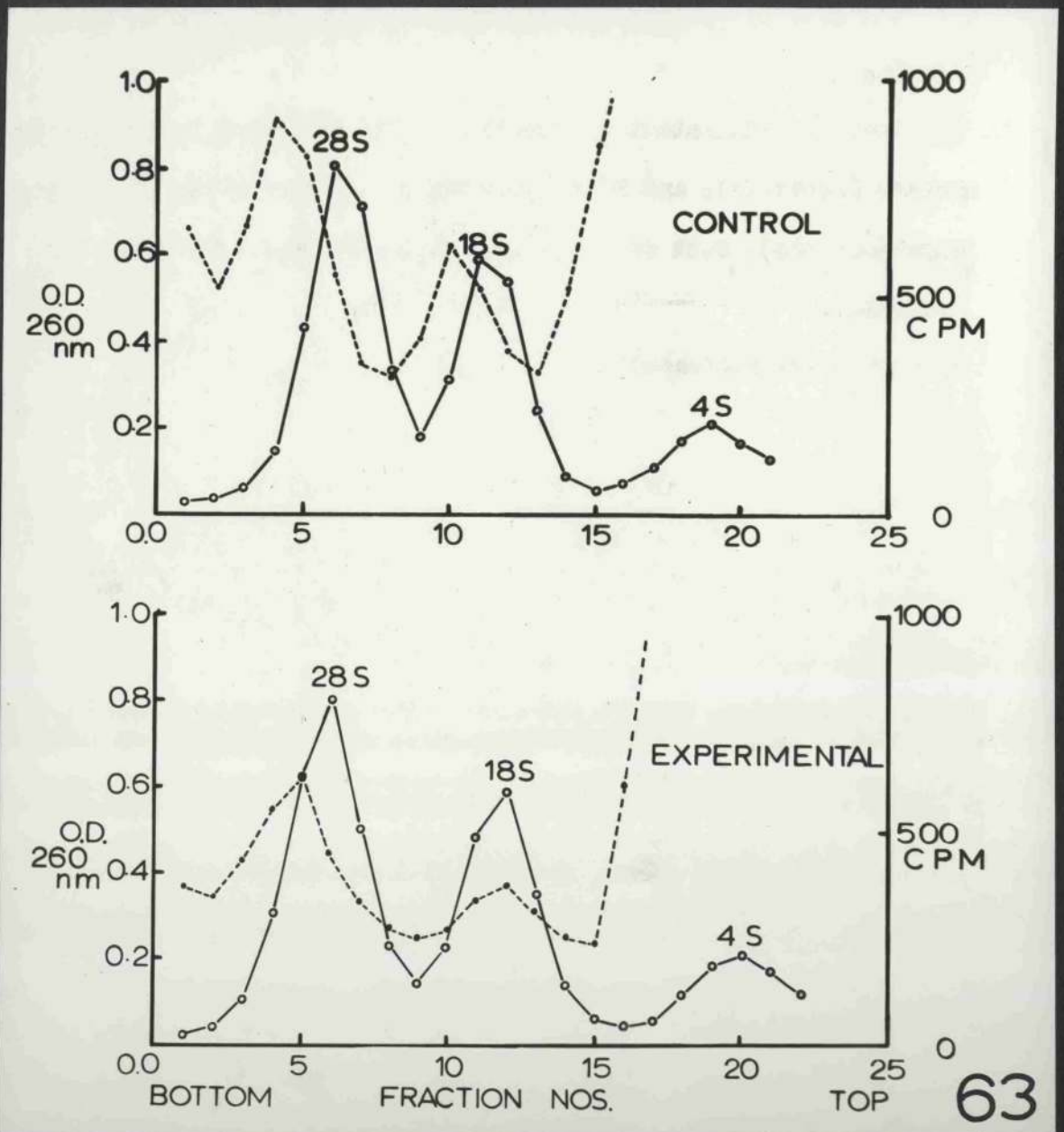
Incorporation of ^{3}H-uridine into RNA of single ovarioles over a period of 4.5h. Each plot is taken from the average count of 10 ovarioles.

Fig. 63.

Sucrose sedimentation patterns of RNA from a) freshly excised ovaries (controls), and b) ovaries which had been cultured for 24h (experimentals), both of which were incubated for a further 6h in ^{3}H-uridine. ●——● represents the O.D. of total RNA. ●- - - -● represents the radioactive newly synthesised RNA.


C.P.M. x10³
60
50
40
30
20
10
0 4 8 12 16 20 24 28 32 36 40 44 48
HOURS

62


O.D. 260 nm
1.0
0.8
0.6
0.4
0.2
28S
18S
4S
CONTROL
1000
500 C PM
0
0.0 5 10 15 20 25
EXPERIMENTAL
BOTTOM
FRACTION NOS.
TOP

63